INFLUENCE OF HEAT, ALUMINIUM TOXICITY AND EXPOSURE TO *BACILLUS SUBTILIS* ON THE GERMINATION OF *ABELMOSCHUS ESCULENTUS*

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DECLARATION

I certify that the work presented in this dissertation is to the best of my knowledge and belief original except as acknowledged in the text and that the material has not been submitted, either in whole or in part, for a degree at this or other universities. I also certify that I have complied with the rules, requirements, procedures and policies of the university.

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To God be the Glory.

DEDICATION

With gratitude I dedicate this work to all those Neo-Einsteinists who are tirelessly pursuing shadows for goodwill to reign amongst humankind.

I am convinced that with your blithe spirit, poverty and sorrow will never reign to let our beautiful beings live without tranquility.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INFLUENCE OF HEAT, ALUMINIUM TOXICITY AND EXPOSURE TO *BACILLUS SUBTILIS* ON THE GERMINATION OF *ABELMOSCHUS ESCULENTUS*

ABSTRACT

Okra (*Abelmuschus esculentus* (L) Moench.) is one of the most popular crops within the Malvaceae family of plants. It is a common vegetable eminently cultivated in regions experiencing constraints to manage climate change. In South Africa climate change coupled with aluminium-enriched soils are responsible to drawbacks crop performance. Therefore, it is worthwhile to whether okra will thrive as an alternative crop in the country. Many studies have identified potential of okra to improve yields of resource poor farmers in Africa. The physiological responses of okra seed to variations in aluminium ions and temperature were not determined. Therefore, a study with okra, cv. Clemson Spineless, seed coated and uncoated with *B. subtilis*, was initiated to assess germination on moist filter paper in 90mm diameter Petri plates. Germination medium consisted of various concentrations of aluminium chloride ($AlCl_3$), 0M, 0.001M, 0.01M, 0.05M and 0.1M. Each aluminium treatment was allocated into incubators adjusted to 22°C, 25°C and 37°C temperatures. This resulted into a 5 x 3 x 2 factorial experiment with five replicates and was conducted in three cycles. Daily scores of germinated seeds were assessed from the second to the fifth day after initiation of germination. During termination, five days after the initiation of the experiment 10 seeds with the longest coleoptiles had their coleoptiles measured using a digital caliper. At the fifth day after initiation of the experiment, coleoptile lengths from 10 seeds per treatment were measured using digital caliper. A total of 50 plates (10 from 37°C in Cycle 1; 30 from 22°C, 25°C and 37°C from Cycle 2; 10 from 37°C in Cycle 3), were selected and germinated were ground and stored at - 20°C before ^{1}H NMR analysis. Metabolites were extracted from 50mg ground seed material with 750 µL methanol-D_4 and 750 µL buffer (deuterium oxide + potassium dihydrogen phosphate). The mixture was vortexed for three minutes, sonicated for 20 minutes, centrifuged at 18000 rpms for 20 minutes and the supernatant filtered through cotton wool. Then the supernatant was dispensed into NMR tubes for further ^{1}H NMR spectroscopic processing using a 600 MHz NMR

Varian spectrometer to generate magnetic spectra of the fifty samples. Results of this study demonstrated that in all the experimental cycles, regardless of aluminium concentration and bacterial seed coating, 37°C inhibited germination percentages and coleoptile lengths in okra seed germination. Germination percentages and coleoptile lengths of bacteria-coated seeds growing in 25°C were most stimulated at all aluminium concentrations, but not at 0.1M. In this temperature germination percentages and coleoptile lengths were highly influenced by the interaction of aluminium concentrations and bacterial coating, respectively. ^{1}H NMR metabolomic association showed no distinct grouping, but clusters across treatments showed to be linked through a subset of metabolites amongst aluminium concentrations, bacterial seed coating and temperatures, respectively. This infers that treatment variations in both seed and bacterial physiological responses were associated through shared metabolic pathways. In conclusion, the study proved that 25°C provide temperature environment within which *B. subtilis* can be able to stimulate growth and remediate physiological constraints from aluminium ions during okra seed germination.

Keywords: Aluminium toxicity, *Bacillus subtilis* metal bioremediation, ionome, metabolome, *Abelmoschus esculentum*, germination physiology.

CHAPTER 1

1. INTRODUCTION

1.1 Background

Okra, (*Abelmoschus esculentus* [L] Moench.) has long been an preferable vegetable in traditional and exotic culinary industry. With the recent statistics showing a significant increase in yield volumes and area of production (FAO: Statistics Division, 2012), it is worthy to consider the potential of okra as an alternative to conventional crops which are challenged to cope with changing environment. Lately, there has been a great interest in okra cultivation with evidence showing more than 70% increase in global production since 1961 to an extend of supporting yields of 8 359 944 tonnes per annum (FAO: Statistics Division, 2012). Beside the statistics it is also important to consider that the crop has long been held as a significant vegetable amongst resource-poor gardeners. Extrapolating from its wide cultivation and genetic diversity in tropical, sub-tropical and warm temperate climatic regions (Benchasri, 2012; Lamont, 1999), it is generally claimed that the crop will adapt in poor to marginal soils and high temperature. However, it is important to validate this claim and determine the potential of the crop to adapt to a wide range of environmental constraints brought by climate change. Heat-stress and aluminium toxicity are amongst some of the most prominent constraints in crop environment leading to yield reduction in conventional crops (Archer and Tadross, 2009; Archer et al., 2008; Kruger and Shongwe, 2004). These constraints were identified as prominent factors of yield reduction in South African corn producing regions (Thompson, 1986). Consequent to that most farmers are shifting their cropping systems to cultivate alternative high yielding crops.

Vegetables are some of the most preferred crops because of their market elasticities and return on investment. With high okra yields it is possible for farmers to scale down their production area and still obtain better returns. However, it is important to consider that there is little trust amongst the farmers to adopt the crop because of poor information which is necessary to guide management practices. Therefore it will be imperative to assess whether okra has the potential to withstand devastating changes in temperature and aluminium conditions.

Generally, heat-stress and aluminium phytoxicity impede physiological processes which are responsible for plants to accumulate photo-radiation and nutrients (Hopkins and Hüner, 2009). Since plants are sessile they have to express specific genetic mechanisms to aid them in resisting or tolerating stresses. From traditional studies morpho-anatomical and low throughput biochemical responses were relied upon to aid biologist in understanding plant stress physiology (Abate et al., 2013; Wahid et al., 2007). With novel omics technologies it is possible to understand biological responses of okra to a variety of growth conditions. These technologies are renowned for their fast capabilities to generate high throughput and reliable information that aids in understanding the genome, transcriptome, proteome, metabolome and ionome of plant. Studies using these technologies rely on model plants, such as Arabidopsis, wheat, maize and cotton, as reference genomes to unravel biological networks responsible for multi-trophic interactions of plants. It is therefore important to generate biological information to determine whether okra is tolerant to heat and aluminium, and more importantly the role of *Bacillus subtilis* on growth and development of the crop. Over and above heat stress and aluminium toxicity, the focus of this study will be *B. subtilis* because this bacterium has been shown to have the ability to assist plants in resisting yield reduction from pests and pathogens. This study will further aim to determine if there are bacterial extracellular factors responsible for ameliorating okra`s physiological injury from heat stress and aluminium toxicity.

1.2 Problem statement and justification

There is paucity of biological information on the responses of okra to heat, aluminium toxicity and exposure of the germinating seed to *B. subtilis*. In light of the need to substantiate the cost effectiveness of the crop as alternative to conventional vegetables prone to climate change, it is important to assess its tolerance to prominent environmental constraints. Global surveys have explicitly determined that increased temperature (Parry et al. 2001, 1999; Rosenzweig et al. 2002, 1995; Rosenzweig and Hillel, 1998; Teixeira et al. 2013) and acid solubilizing soil aluminium (Von Uexkull and Mutert, 1995) have negative impact on yields of established crop genomes. The mentioned factors are capable of causing drastic

physiological absurdness of different crops, including in some cases wiping out of newly introduced crops. With food security under threat the experience is imposing enormous pressure on plant biological research teams to identify cost effective solutions. In this rather cumbersome quest some of the principal accounts have pinpointed the need to improve technical repertoire of experimental technologies. As a result, biological research is expected to make use of regimented and high throughput technologies to fast-track our understanding of whole genome biology of neglected food crops.

Okra is extrapolated as been tolerant to a wide range of environmental stress factors because of its genetic diversity in regions with tropical, subtropical and warm temperate climates (Charrier, 1984). However, it has been established that the crop shows poor, if not slow, germination and seedling establishment under field conditions. This problem has been attributed to its hard seed coat which deprives oxygen diffusion and flushing of anti-germination metabolites. Furthermore, erratic germination and poor seedling establishment were shown to be induced by variation of temperature level and moisture availability (Sionit et al., 1981). It is also suspected that inconsistencies observed in seed germination and seedling establishment of okra might be as a result of different stages and evolution of morphological traits from environmental pressures. Regional temperatures have a significant pressure on ecophysiological traits such as dormancy and germination (Hoey and Parks, 1991) and morphological traits such as embryo growth (Baskin and Baskin, 1998). Beside temperature, aluminium toxicity is one of the most common abiotic stresses that were never studied in okra germination. In this regard, it will be important to assess physiological responses of okra seed germination under different temperature and aluminium thresholds. Moreover, the bioremedial properties of *B. subtilis* will help to identify whether the bacterium has the potential to shield the germinating seed from heat stress and quench aluminium toxicity. With the use of ^{1}H NMR metabolomics, metabolomic shifts in temperature, aluminium and *B. subtilis* treatments of the germinating seed will be determined.

1.3 Aim and objectives

1.3.1 Aim

The aim of the study was to assess the germination responses of okra to heat, aluminium toxicity and exposure of the germinating seed to *B. subtilis.* Various tools to achieve this aim employed germination assays and assessment of the chemistries of the treatments.

1.3.2 Objectives

Objective 1: Assess phenotype responses (seed germination) in okra over the interaction of heat, aluminium toxicity and exposure of *B. subtilis*.

Objective 2: Assess molecular responses in okra over the interaction of heat, aluminium toxicity and exposure of the seed to *B. subtilis*.

1.4 Reliability, validity and objectivity

The degree of credibility in scientific research relies on procedures and instruments applied to generate information and data analysis of biological modalities pertaining to the research question of interest. In this view, it is important to make use of reliable, valid and fair methods in establishing and managing experiments, and also most importantly recording data with highest levels of precision possible. Reliability refers to the trustworthiness of instruments applied in assessing biological responses to yield consistent results (Drost, 2011). The fit-for-purpose approach was adopted in this study to use an *in vitro* bioassay to assess germination. The resultant data was analysed using suitable algorithms to draw conclusions. Validity is concerned with determining whether the research techniques applied in generating data are relevant to explain the biological responses encountered during the course of the experiments. In this study, factorial experiments were repeated three times under similar controlled conditions. Furthermore, a control treatment was equally replicated within the experiments to ensure that variance encountered from the studies is

initiated by variable treatments. Objectivity refers to applying research methods which avoid biasness, preconception and subjective evaluations in empirical studies (Leedy and Ormrod, 2005). The findings of the study were discussed in reference to statistical analyses and biological trends were associated with verdicts from previous empirical studies.

1.5 Bias

In the management of experiments it is important to use strategies which limit biasness. Bias can be defined as any tendency which prevents prejudiced and subjective evaluations of biological responses in experiments. Bayona and Olsen (2004) defined bias as an error in design or execution of the study which produces results which are distorted in one direction because of non-random factors. In this study, bias was minimised by ensuring that the experimental error in each experiment was reduced through increased replications, randomisation and repetition of experiments (Little and Hills, 1981).

1.6 Significance of the study

The study was intended to generate high throughput baseline biological information to enable understanding of okra germination responses to heat, aluminium toxicity and exposure of the germinating seed to *B. subtilis*. In view of guiding mass commercialization of the crop in South African growing conditions it is important to anticipate the effects of heat-stress and aluminium toxicity in okra`s growth and development processes. Interaction of these factors exhibit growth inhibition (Craufurd and Peacock, 1993; Savin and Nicolas, 1996). For example, abnormal temperatures and soil acidity causes more yield reduction to South African corn industry (Thompson, 1986). Besides, a greater shift toward the use of tolerant cultivars some farmers are considering neglected crops such as okra to avoid losses in investments. However, with limited information on the tolerance thresholds of okra to environmental stress factors most farmers remain sceptical to adopt the crop. Therefore, providing information on the germination response of okra to heat and aluminium stresses may increase the farmer’s confidence in adopting the crop, if

okra is tolerant to the harsh conditions of aluminium toxicity and heat stress, as compared to some conventional vegetable crrops. Furthermore, the use of environmentally friendly agents such as *B. subtilis* is gaining momentum in this new age agriculture which is in one way or the other faced with consumer sceptism. *B. subtils* is a well known soil microbial resident with significant role in improving soil conditions for plant growth. Moreover, the use of the soil-abundant *B. subtilis*, in reducing the effects of these stresses may provide a new twist in the okra agronomy.

In South Africa, the booming markets for West African food, which okra is an important ingredient, and high numbers of immigrants justifies the market potential for domestic production. As such, it is important to assess tolerance of okra to avoid unanticipated yield losses. The crop is received as a treasure because when compared to conventional cultigens it can grow in marginal environments characterised by heat stress, aluminium toxicity and various other stresses. Nevertheless it is misleading to consider the crop as adaptable to other countries without empirical evidence. In this case it is imperative for research to identify relief mechanisms such as resistance or use of eco-friendly bacteria.

1.7 Format of dissertation

In this ending Chapter 1, the over-arching motive to conduct this study was explained and it will be followed by literature review in Chapter 2 and 3 which appraises the work done on physiological responses of plants to heat-stress, aluminium toxicity and exposure of *B. subtilis*. Then, in Chapter 4 subsequent methods used in conducting experiments and analysing generated biological data will be elaborated; which ends with reporting respective results of the study. Lastly, findings, conclusion and recommendations will be discussed in Chapter 5.

CHAPTER 2

2. LITERATURE REVIEW

2.1 *Abelmoschus esculentus*: Description, markets and agronomic management

2.1.1 Description

Okra (*Abelmoschus esculentus* [L] Moench) is commonly known as bhindi (India), krajiab kheaw (Thailand), ochro/okoro/quimgombo/quingumbo/gombo/kopi arab/kacang/bendi/bhindi (South East Asia), bamia/bamya (Middle East), bamieh/gumbo (Southern USA), ladys finger (England), quiabo (Portuguese and Angola), quimbombo (Cuba), gombo commun/gombo/gumbo (France), mbamia/mbinda (Sweden), okura (Japan) or qui kui (Taiwan) (Chauhan, 1972; Lamont, 1999; Ndunguru and Rajabu, 2004; Siemonsma and Kouame, 2000). The crop is botanically classified within the Malvaceae family which also classes economic crops such as *Gossypium* species, *Ochroma pyramidale*, *Hibiscus cannabinus*, *Ceiba* and *Bomax* species for timber-fibre products; and food crops including *Theobroma cacao*, *Cola nitida*, *C. acuminata* and *Durio zibethinus* (Dempsey, 1975; Judd et al., 2008). It is an annual herbaceous, shrub-like dicotyledonous vegetable popularly cultivated under monoculture or intercropping systems in tropical, sub-tropical and warm temperate regions (Emuh et al., 2006; Mohammed, 2010). Besides its immature pods it can also be cultivated as a biennial to serve as a fiber crop for industrial uses. The stems of okra are woody toward the base; are erect and hairless to bristly-hairy growing up to 1.5 m or more to support corpulent and branched plant architecture. Leaves are palmately fivelobe shaped to compound or scarcely lobed, long-petioled and 6-35 cm wide with alternate arrangement along the stems. During reproductive stage it develops inflorescence of solitary, large, stout-pediceled flowers in the upper leaf axils, involucral bracts 8-12, linear and with 2.5 cm in length. The plant generally develops large radially symmetric and broadly bell or funnel-shaped flowers with calyx having white to yellow, purple or red highly defined at the base while faintly evolving to the posterior flower ends. Flowers grow up to 7.5 cm wide with capsules: many-seeded, 5-angled,

6-25 cm long, cylindrical or finger-shaped, beaked, bristly then nearly hairless, green to purple when young and brownish when mature. The flowers are classified as self-fertiles (hermaphrodite) expressing levels of allogamy of up to 63% (Martin, 1983). These flowers progress growth into oblong (in cultivar: Clemson spineless) or round (in cultivar: Gomba) pods which during drying yield blackish globular seeds (3-6 mm across). The pods are smooth to ridged and light green, green or purple in colour. It is important to note that the flower is an important structure for the classification of *Abelmoschus* species.

Considering challenges with botanical classification techniques okra was formerly classed within the *Hibiscus* genus because its flowers resemble those of common *Hibiscus* species. After thorough examination of floral structures it was moved to an independent *Abelmoschus* rank, which was then raised to be a distinct genus by Medikus in 1787 (Aladele et al., 2008). Currently greater number of *Abelmoschus* species and breeding lines are classified through the use of pollen (P) and ovule (O) scores to correlate morphological traits (Cruden, 1977). Scores of P and O are used in Cruden index: log (P/O), evaluation scale of which runs from 0.65 (cleistogamy) to 3.65 (xenogamy) is used to classify *Abelmoschus* species. Although this index was developed for use during the olden days, it also serves its purpose in plant breeding evaluations of modern days. In Harmon and Koechlin (1991), Cruden index was used to determine variation of P and O scores in the breeding system with accessions of four *Abelmoschus* species: (1) cultivated, *A. esculentus* and *A. caillei*; and (2) wild, *A. moschatus* and *A. manihot*. Cultivated species exhibited identical averages (2.0 and 2.05) in the same way as in wild species (2.17 and 2.19), with less pollen grains per anther in *A. esculentus* (72) than *A. moschatus* (203).

Furthermore, studies have shown that *Abelmoschus* species have a wide cytogenetical variation. In Datta and Naug (1968) it was been suggested that the numbers of chromosomes in *Abelmoschus* species vary with regular multiples of 12 giving a series of polypoids such 2n = 72, 108, 120, 132 and 144. However, there are unsatisfactory taxonomic classifications at the species level with the highest number of chromosomes reported in *A. manihot var. caillei* (200) and *A. angulosus* (2n=56) having lowest chromosome numbers (Ford, 1938; Siemonsma, 1982). The most frequently observed somatic chromosome number of okra is 2n = 130

(Benchasri, 2012). Despite having poor empirical information in this field it is suggested that Asian okra material and related species are likely to provide more examples of the existence of amphidiploids in the *Abelmoschus* genus (Siemonsma, 1982). The origin of *A. esculentus* is argued to be traced from either *A. tuberculatus* or *A. ficulneus*; putative ancestors native to Northern India and East Africa respectively (Bencharsi, 2012). In some accounts it is alleged to originate from Ethiopia where it was subsequently spread into massive cultivations in Egypt, North Africa and Middle East (Lamont, 1999; Saifullah and Rabbani, 2009; Tindall, 1983). Apart from *A. esculentus*, *Abelmoschus* genus constitutes other species such as *A. moschatus, A. manihot, A. tuberculatus, A. ficulneus, A. crinitus* and *A. angulosus* (Charrier, 1984) which thrive under a wide variety of crop environments in tropical, subtropical and warm temperate regions (Bencharsi, 2012).

2.1.2 Markets

Okra is largely produced for its fresh pods and secondarily valued metabolic constituents in food derivatives and pharmaceuticals markets. Production and yield have been increasing over the years. In the 1991/1992 growing season, total area and yield were recorded at 0.22 million hectares and 1.88 before increasing to 0.396 million hectares and 4.07 million tons in 2006-2007, 0.43 million hectares and 4.54 million tons in 2009-2010 (Benchasri, 2012; Burkil, 1997). Recently, Varmudy (2011) showed that okra is produced from a total area exceeding 1.02 million hectares, providing over 6 million tons in yields. Furthermore, a recent study showed that India and Nigeria were the biggest producers in terms of cultivation area and yields; constituting 67% and 15% of global production, respectively (Varmudy, 2011). However, other countries contribute high yield volumes to the global markets which are encouraged from increased productivity rates (as shown in Table 2.1). In Food and Agriculture Organization: Statistics Division (2012) it is demonstrated that major okra producing countries produce yields lower than 7 704 kg per hectare which justifies the need for research focus on the crop agronomic management.

Table 2.1: Global area, yield and productivity per hectare of okra in 2008 – 2009 production seasons. Data sourced from Varmudy (2011).

Country	Area in ha	Yield in MT[a]	MT/ha[b]
India	432 000	4 528 000	10.5
Nigeria	387 000	1 039 000	2.7
Sudan	21 926	223 650	10.2
Iraq	22 250	141 000	6.3
Ivory Coast	46 000	115 867	2.5
Pakistan	15 081	114 657	7.6
Ghana	19 500	108 000	5.5
Egypt	6 800	107 000	15.7
Benin	13 658	48 060	3.5
Saudi Arabia	4 000	46 000	11.5
Others	58 365	276 206	4.5
Total	1 024 580	6 749 440	6.6

[a] = yield of okra in metric tons; [b] = MT/ha refers to metric tons per hectare which is a measure of productivity in land use relative to yield volumes.

Generally young immature okra pods are consumed as fresh fruits in different forms varying from boiled, fried and cooked dishes (Akintoye et al., 2011; Ndunguru and Rajabu, 2004). Okra pods contain proteins, carbohydrates and vitamin C making the okra crop an important ingredient for addressing human nutritional deficiencies (Arapitsas, 2008; Dilruba et al., 2009; Gopalan et al., 2007; Kahlon et al., 2007; Lamont, 1999; Owolarafe and Shotonde, 2004; Saifullah and Rabbani, 2009). In Thailand, the pods are usually boiled in water resulting in slimy soups and sauces which are a custom for Thai people, and therefore making the fruits an important highly valued thickener for both confectionary and catering industries. Moreover, the plant possesses superior nutritional attributes. Fresh okra pods, which are peaked during their immature state, have been associated with high levels of vitamin C (59 mg), calcium (532 mg), phosphorus (70 mg), iron (0,7 mg), antioxidant activity (3968 µmol ascorbate equivalent/100g fresh weight) (Gebhardt et al., 2004; Gopalan et al., 2007); and most importantly novel pharmaceutical properties such as cholesterol-

lowering effects (Jenkins et al., 2005) and suppression of ulcer-inducing *Helicobacter pylori* in the human gastrointestinal tract (Lengsfield et al., 2004). Furthermore, 100 g of edible leaf parts contains 81.50 g water, 81.50 g energy, 4.40 g protein, 0.60 g fat, 11.30 g carbohydrate, 2.10 g fibre, 532.00 mg Ca, 70.00 mg P, 0.70 mg Fe, 59.00 mg ascorbic acid, 385.00 µg β-carotene, 0.25 mg thiamin, 2.80 mg riboflavin and 0.20 mg niacin (Gopalan et al., 2007). It has been observed that the nutritional composition of pods changes when fruits are kept for longer on the plant before harvesting. The pods are said to accumulate more anti-nutrient factors. Young pods and leaves are rich in mucilage, and young fruit has high antioxidant activities. Liu et al. (2005) showed that mucilage is a carbohydrate making okra slimy when cooked because of its resistance to flow like water (30% viscosity). This water-soluble carbohydrate is made of galactose (25%), rhamnose (22%), galacturonic acid (27%) and amino acids (11%). Subrahmanyam et al. (2011) also showed that the pod of the crop has antidiabetic activity.

2.1.3 Agronomic management

Data that describe the management strategies of okra is scanty. In okra production seeds are directly sown into well tilled soils when ambient temperatures are between 30 and 35°C (Akande et al., 2003). To soften the hard coat, okra seeds are soaked in warm water overnight to enhance germination (Splittstoesser, 1979). The crop performs well in well-drained sandy to loam soils with a neutral pH and high organic matter content (Adilakshmi et al., 2008; Lamont, 1999). The okra plant forms a deeply penetrating tap root with dense shallow feeder roots reaching out in all directions in the upper 0.45 m of soil. During its growing period of October to April month's okra requires a moderate rainfall of 80-100 cm well distributed to produce its young edible fruits over a relatively long period. At temperatures below 30°C it is preferable to use transplants to avoid delayed harvest periods. The crop needs more than 20°C ambient temperatures for optimum growth and development in both vegetative and reproductive stages (El-Kader et al., 2010; Lamont, 1999). Short days are important for flower initiation and flowering. However, a range of cultivars have been developed to cater for its cultivation in broad geographic distribution with varying day lengths of photoperiod. These cultivars express variable sensitivity to

day-length with most local cultivars showing critical day-length of 12.30 hours. This explains why flowering of inland cultivars of common okra is only quantitatively affected by day length in the coastal areas of the Gulf of Guinea (5°N) (Benchasri, 2012). The crop is highly sensitive to terminal low and high temperatures (Akinyele and Temikotan, 2007; Dada and Fayinminnu, 2010). High temperatures during the on-set of flowering lead to abortion of flowers, and therefore yield reduction. Besides the importance of agronomic inputs and cultural management of the crop, abiotic stresses such as heat and soil aluminium toxicity play a critical role in determining pod production and yield. Also interesting, is the use of growth promoting bacterial for optimizing the soil microbial functions of the crop rhizosphere with the aim to suppress disease inducing organisms and plant nutrient assimilation. *Bacillus subtilis* has, in recent years, gained prominence as a plant growth promoter and control agent against fungal diseases of plants. *B. subtilis* has been shown to be a good soil bioremediator (Han et al., 2014).

2.1.4 Objective of the literature review

In view of introducing okra in South Africa it will be important to draw experiences from countries with well established okra production. The purpose of this literature review is to highlight existing poor knowledge about germination of okra in response to heat, aluminium concentrations and exposure of the germinating seed to *B. subtilis*. Given the potential of the crop in improving rural livelihoods it is further important to consider the capacity of research technologies to develop genotypes which requires less inputs. As such the literature review will focus on physiological responses which were previously studied to appropriate plant resistance to heat and aluminium stresses. Furthermore, the role of bioremediators such as *B. subtilis* will be elucidated with reference to their ability to remediate physiological stress in plants. Also, low and high throughput technologies will be compared to show inconsistencies between the technologies in understanding multitrophic interaction between temperature, aluminium and *B. subtilis* in plants.

2.2 Assessing heat-stress in plants: Low throughput studies

High ambient temperatures of the crop environment cause energy gradient between the plant bodies and atmospheric continuum (Berry and Raison, 1981). In this regard, as explained through thermodynamic principles, energy flows towards plant bodies in the form of heat, thereby creating higher ionization states of biomolecules in plants (Berry and Raison, 1981; Dragicevic and Sredojevic, 2011). As a result of these ionization states the plant foliage accumulates temperatures higher than ambient temperatures. With climate change this condition has turned the crop environment into ovens of unforgiving heat pulsation. The condition threatens sustainable production of a lot of conventional crops which unlike hardier crops such okra have been genetically fine tuned into cultigens. In cultivation of cultigens it has been found that their fitness to the harsh heat levels is dependent on cost intensive man-made measures to reduce stress levels, which in some cases confine productions exclusively into indoor environment. This is because high heat levels lead to high levels of transpiration which are mainly intended to cool off leaves back to optimum temperatures which do not inhibit photosynthesis (Kumar et al., 1994). Okra thrives in regions with wide climatic variation and it is expected that it will tolerate variable heat thresholds. As such, it is important to shed focus on the effects of heat stress in plant growth and development.

2.2.1 Morpho-anatomical and phenological responses

2.2.1.1 Morphological-anatomical responses

Plants growing under stress over prolonged periods tend to exhibit observable changes in whole plant stature. Temperatures above thresholds for optimum physiological processes of plants constitute heat-stress that is directly perceived by the above-ground components of the plants. Similar to drought, which varies in concert with temperature perturbations, heat-stress causes excessive dehydration of plant cells with subsequent stunted growth of plants. These conditions are further exacerbated by high levels of radiation and drought in tropical and sub-tropical climates. With the majority of plant metabolic activities undertaken under aqueous

conditions dehydration inhibits primary processes of carbon assimilation resulting in reduced biomass production and yield.

Successful adaptation of terrestrial plants to heat stress and other abiotic stresses is largely, if not exclusively, dependent on the functions of the water conducting system. Two kinds of conducting tissues are represented in this system; the xylem for the purpose of conducting water with nutrients from the soil and the phloem serving as a conduit of photoassimilates. In the event of terminal temperatures plant develops greater xylem vessels in both root and shoot, hence, altering the trade-offs between net photosynthesis and transpirational rate. Higher water use efficiency in heat-stress intolerant crops is substantiated by longer xylem vessels anatomy which is intrinsically developed to succumb to increased transpirational rates. In an attempt to subdue increases in transpirational rates the plant resorts to mechanisms leading to the reduction of photosynthetic surface of the crop canopy. As a result, plants shed more leaves to reduce canopy size, leaves fold in-ward to protect structural integrity of membranes, internodes shortens in length to reduce metabolic requirements in growth and maintenance, and senescence sets earlier with ineffective reproductive structures than expected. These plant`s anatomical changes in response to heat-stress have been consistently used in traditional studies because of their ease to detect and quantify. However, it is important to bear in mind that observable changes in plant anatomy are preceded by a diversity of biochemical changes.

2.2.1.2 Phenological changes

Crop yield is directly influenced by accumulative increase in photoassimilates throughout different growth stages. In this regard, temperature environment is important to synchronize physiological transitions between growth stages in response to environmental changes. Through thermal kinetic window values Burke et al. (1988) displayed an inherent biochemical trait through which ambient temperature regulates plant phenology. Thermal kinetic window is described as the relationship between temperature changes and activity of glutathione reductase isozymes. It is from this approach that Haldimann et al. (2005) and Moore et al. (1998) found that leaf temperatures tend to exceed ambient temperatures by as

much as 10°C under field conditions. Coupling foliage temperature measurements with kinetics of glyoxylate reductase for NADH in cotton and wheat exhibited a linear relationship between the duration of foliage temperatures within thermal kinetic window and biomass production (Burke et al., 1988). The study further showed that a crop estimated to have thermal kinetic window of 30% reflect that about 70% of the growth season is required to reach maturity. In spite of being easily applicable in daily crop management programs, thermal kinetic window was the first index to pinpoint biochemical perturbations of plants in relation to changes in ambient temperature on a macro environmental scale. Consequently, growth phases have to be shortened because of restrictions in water changes. In case of okra, it was observed that different genotypes vary in response to temperature (Marsh, 1992). As such, it will be important to further prospect okra genotypes for any novel genes.

2.2.2 Physiological responses

2.2.2.1 Cellular water potential

Heat stress increases the plant cells affinity to water. Different states of dehydration, similar to those in water deficit treatments are observed in plants growing under high temperature conditions. Machado and Paulsen (2001) demonstrated that plants supplied with optimum water tend to maintain tissue water status, whereas heat-stress accompanied with limited water severely impairs water status in plant growth and development. Several crops exhibited impairment in translocation and transpiration responses when exposed to heat-stress conditions (Ãnon et al., 2004; Morales et al., 2003; Wahid and Close, 2007). These results show that plant physiological functions are dependent on cellular water status. Water-starved cells are unable to modulate competition between translocation and transpiration processes. In Rieger (1995), it is suggested that stomatal closure and osmotic regulation are critical mechanisms in offsetting homeostatic imbalances caused by heat stress. Exposing okra seeds to 15°C and 35°C temperature significantly decreased the content of reducing sugars while supporting accumulation of high potassium levels (Besma and Mounir, 2010). These temperature levels are

acclaimed as the minimum and maximum thresholds within which okra seed and seedling growth is optimum. However, it is has been demonstrated that even though the crop is adapted to regions with wide spectrum temperatures ranging from tropical to Mediterranean climates (Momo, 2014); it cannot resist growing at 40°C (Besma and Mounir, 2010). This behavior might be as a result of its limited vessels and fibers in the root and leaf xylems (Nwachukwu and Mbagwu, 2006). Xylem tissues play a crucial role in asserting plants conduction of water from the soil in order to correct foliar water deficit. Collapsed xylem in Arabidopsis phenotype was associated with poor cellulose deposition in secondary cell wall (Turner and Somerville, 1997) which translates to the inability of plants to withhold compressive force of water imposed toward transpirational flow. This weakened xylem structure disposes phloem tissues with constraints to phloem-xylem patterning. Furthermore, it is important to consider that some plants have a tendency to offset mechanical strain through circumventing low hydraulic conductance with accumulation of osmolytes (Hare et al., 1998; Mashela and Nthangeni, 2002; Sakamoto and Murata, 2002). Nonetheless, it will be worthwhile to consider substantiating this theory with experimental evidence before drawing conclusions.

2.2.2.2 Photosynthesis

Yield biomass of crop plants is dependent on the leaf anatomy and metabolic fate of fixed atmospheric CO_2 during photosynthesis. Okra leaf-type anatomy has been a much sort after source of superior traits for cotton breeding programs (Wells et al., 1986). In Pettigrew et al. (1992; 3), super-okra and okra isolines were 42% thicker than normal cotton leaf-types, with subsequent increased leaf chlorophyll concentration, CO_2 fixation and water use efficiency. The results of these studies anticipate that genotypic variation amongst related plant genomes can be exploited to improve genetic weakness in the other. Genotypes with superior photosynthetic rates were identified and cross-bred with those accessions with better partitioning of photosynthates between reproductive and vegetative growth (Pettigrew et al., 1992; Wells and Meredith, 1984). This relationship between anatomy and metabolism of leaves proves to determine the ability of plants to tolerate heat-stress. Plant genotypes with sparse canopies have the most foliage to support increased

transpiration. During exposure to high temperatures plant photosynthesis is inhibited as a result of suppressed Rubisco activase and Rubisco activation which are considered to play a decisive role in carboxylation reactions in which CO_2 is added to ribulose-1,5-bisphosphate to form a transient and unstable six-carbon intermediary enzyme (Feller et al., 1998). Rubisco, with high affinity to CO_2 than oxygen is optimally activated at temperature thresholds lower than 37°C (Hopkins and Hüner, 2009; Salisbury and Ross, 1991). In the event of abnormal thresholds, Rubisco is spontaneously deactivated, which consequently leads to reduced foliar carbon metabolism in HS-intolerant genotypes (Demirevskaa-Kepova and Feller, 2004; Hikosaka et al., 2006; Salvucci and Crafts-Brandner, 2004a,b,c; Sharkey, 2005; Spreitzer and Salvucci, 2002). Thermotolerant plants exhibit an associative response in which Rubisco is reactivated by Rubisco activase (Salvucci,1993), which is a nuclear encoded protein that is transported to the chloroplasts where it catalyzes the activation of Rubisco (Santa Cruz Biotechnology, Incorporation)..

2.2.2.3 Assimilate partitioning

The primary function of photosynthesis is to provide energy and carbon sufficient to support maintenance and growth, without singling out other plant growth components (Hopkins and Huner, 2009). During heat stress the transfer of carbon between sources and sinks is altered leading to severe reductions in growth, economic yield and harvest index (Wahid et al., 2007; Wahid and Shabbir, 2005). As output of photosynthesis, photoassimilates in the form of sucrose and glucose must be translocated from the leaves to the roots. Under low to moderate heat stress, a reduction in source and sink activities may occur. However, considerable genotypic variation exists in crop plants for assimilate partitioning, as for example among wheat genotypes (Yang et al., 2002). This indicates that the quantity of partitioned assimilates is dependent on photosynthetic fixation of atmospheric CO_2 and mobilization of structural sugars, respectively. As a result, over the year's crop breeding programs have been relying on differences in radiation use efficiency, stomatal conductance and canopy size (Cornish et al., 1991; Radin et al., 1994; Lu et al., 1994; Lu and Zeiger, 1994). To date, a great emphasis is put on those genotypes distributing better fractions of their carbon biomass between reproductive

and vegetative growth structures. To elucidate causal agents of reduced grain filling in wheat under high temperatures, Wardlaw (1974) examined three main components of the plant system including source (flag leaf blade), sink (ear), and transport pathway (peduncle). The results showed that photosynthesis in wheat had a broad temperature optimum from 20°C to 30°C; however it declined rapidly at temperatures above 30°C. In maize, heat-stress has a dire effect on tassel-silking synchronization, also known as anthesis-silking interval, with visual effects including short tassels, less number of branches and weak anther bearing spikelets (Cicchino et al., 2010). With regard to okra it has been shown that water deficit deems to aggravate heat-stress effects on the total yield (Gunawardhana and De Silva, 2011), which might translate that the crop demands regular cooling of leaves in times of heat spells. Nonetheless, this insight is yet to be empirically proven, and it might help in understanding the mechanisms responsible for okras adaptation to a wide range of climatic conditions. In the cause of exploiting the crop it will be important to prospect the availability of novel genes from the farmer landraces.

2.2.2.4 Cell membrane thermostability

The degree of the fluidity of the membrane in plants growing in stressful conditions plays an important role for inferring tolerance mechanism. As the initial site of signal perception membranes have to avoid leakage of osmolytes, failure to which imposes plants to invest more for metabolic energy generation. The role of cell membrane stability in ion accumulation of okra in response to salts and salinity was determined in Ashraf et al. (2003). Unfortunately, the results were inconclusive to provide an understanding of the whole biological responses of the membranes in the plant. Nevertheless, a diversity of cell wall polysaccharides such as galactose, rhamnose and galacturonic acid have been characterized from okra pods in association to disease resistance of the plant (Sengkhamparn et al., 2009a,b). Furthermore, the study showed high degree of acetylation than methyl-esterification. Decreased degree of polysaccharide acetylation in transgenic Arabidopsis and *Brachypodium distachyon* plants was correlated with increased resistance to pathogens (Pogorelko et al., 2013). Silencing of cinnamyl alcohol dehydrogenase in *Nicotiana attenuate* was reported to block lignification with subsequent delay and restricted spread of red

stem pigmentation (Kaur and Gupta, 2005). In conclusion, it will be important to determine the biochemical functions of lignin, suberin and cutin polysaccharides in protecting okra from cellular water dehydration. These structural saccharides control the movement of gases, water and solutes throughout the plants conductive tissues.

2.2.3 Ionomics and related biochemical responses

Biochemical changes always precede the visible morpho-anatomical modifications of plants when exposed to environmental stresses. Temperature variations induce biochemical changes allowing induction of modulatory reactions which play a primary role in attenuating homeostatic balance. Plant nutritional homeostasis intrinsically dependent on changes environmental growth factors. In the advent of computational biology high throughput technologies can be exploited (Van Baxter et al., 2008; Salt, 2004). As such in complementing other '*omes*', ionome was first described in Lahner et al. (2003) to summate metals, metalloids and non-metals in living organisms, thereby evolving metallome (Outten and O`Halloran, 2001; Williams, 2001; Szpunar, 2004) to include biologically significant non-metals such as N, P, S, Se, Cl and I. This was made possible when Inductively Coupled Plasma – Mass Spectroscopy (ICP-MS) was up-graded with features to generate genome-wide profiling of nutrients and trace elements (Lahner et al., 2003). This ion profiling strategy is acclaimed for its robustness and scalability into parallel or massively parallel analytical technique, with the capabilities to the capacity to analyse 1000 plant samples (root, leaf or seeds) per month, on average. Ionomic-variant plant populations (wild and/or transgenic) are cultured side-by-side under homogenous growth conditions, including uniform growth media.

When conducting these experiments cautions must be considered not to make use of metal materials when tendering the plants, to avoid introduction of unperceived metals into the plants. Concurrently grown to similar size, plant batches per treatments must be harvested under sanitized conditions using metal-free tools and measuring equipment. Equivalent samples must be collected from the plants and washed to remove any surface contaminants, dried, weighed, and analysed accurately and precisely. Elemental analysis is conducted through the use of ICP-MS with 3/4 and 6/7 orders of magnitude and capabilities to detect quantification of

multiple elements simultaneously from small samples. Other than ICP-MS, other studies used Atomic Absorption Spectroscopy (AAS) and Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). Given the volume of samples in thousand orders collected data must be stored into secure databases for future processing and data analysis.

Pursuing ionomics in metal-toxicity growth studies will aid in substantiating von Liebig's Law of the Minimum, which states that deficiency in one of the nutritive elements results into diminishing marginal returns in plant growth regardless of optimum assimilation in other nutrient elements. Certainly in crop agronomic practices, it has been observed that resorting to the best crop nutritional programs does not account to total return on investments, in any actual cases whatsoever. This has been accounted to a blend of other natural factors beyond control by man, however, still similar account pertains also in cases with in-house agriculture, where weather parameters are controlled and programmed for any weather variations. Ionomic data will avail simplicity to develop mapping of those traits describing genomic efficiency specific to various crop genotypes in any given environment, no matter the stress factors. Most important in plants ionome, is their membranes specific selectivity to nutrient factors under various optimal and sub-optimal growth conditions. As such, ion transporters, multiple genes and gene families have been identified in various plants for acquisition of Fe, Zn, Na, P, K and Ca (Curie and Briat, 2003; Mäser et al., 2001; Rausch and Bucher, 2002;Zhu, 2003). However, it must be acknowledged that the number of ion transporters characterized is insignificant to provide genome-wide ionomic variation (Salt, 2004). In the Arabidopsis genome about 1 000 ion transporters were expressed, most of which have not been characterized. Also, out of 25 000 genes in the Arabidopsis genome 5% were reported to be involved in regulating the ionome (Lahner et al., 2003). This procedure stands a good chance to be interfaced with modules sensitive to profile stable isotopes of non-metal and metal elements in living systems.

2.3 Aluminium toxicity in plants

Worldwide soil acidity has been a contentious field of research especially since the advent of the plant nutritionist's curiosity to eliminate soil phenomena that imposes plants into nutritional deficiencies. Aluminum is one of the stand-alone metal-toxins encountered by plants in soils. This metal is associated with its ubiquitous nature as primary build-up of soil mineralogy in the form of Ca^{+2}/Mg^{+2}/Fe^{+3}-silicates (Delhaize and Ryan, 1995; van Rensburg et al., 2009). Also, the metal is dominantly associated with acid soils which are a common challenge to agriculture worldwide. This nutrient-locking phenomenon has been observed in soils surveyed in the Eastern high-rainfall region of South Africa (Beukes, 1995). The toxicity of aluminium in plant can be explained through the hypothesis of lyotropic series, which states that multivalent (3^{+}) are strongly retained onto soil colloids than monovalent (1^{+}) metal-ions. In this regard less than micro-molar concentrations of trivalent aluminium ions have the greater affinity to plant membranes than other primary nutrients. As a result soils enriched with aluminium exhibit deficiencies of Ca, Mg, Mo and P implying farmers with two-fold strategy, either to supplement with nutrient fertilizers or adopt acid/Al-tolerant crop genotypes, in order to increase yields.

Over the years it has been realized that winning the war over lowering levels of hydrogen potential deserves less price if the battle does not lead to reduced levels of high affinity metals like aluminium. This has become more precedent with the consideration that the two phenomena have a direct relatedness in further locking essential plant nutrients such as calcium and potassium onto the soil colloids. Toxicity of the aluminium metal in biological systems can be understood when considering that most organisms evolved from environments with almost neutral levels of hydrogen potential. As such, a limited number of conventional crops, majority of which belong to the Graminaneae family, are able to tolerate or adapt to low hydrogen potential of soils. Even though there is limited information on the biological functions of aluminium ions, this metal modifies transport functions of membrane phospholipids, disrupts functions of transporter proteins, and substitutes Mg^{2+} within biological diverse ligands. Also, trivalent aluminium ions have the greatest affinity to negatively charged plant root membranes. This explains the causes of severe damage and death of the roots in plants which in some cases shows symptoms of nutrient and water deficiencies. Several conventional crops, in

particular from the Grammineae family, have shown to thrive under high levels of soil aluminium with some findings associating their tolerance with environment-induced mutations. These mutations were to some extent justified by the views that the majority of grasses tolerate aluminium because of their consistent production in regions striped by soil acidity and high aluminium levels. Aluminum resistance in plants is either extracellular or intracellular. Intracellular resistance is expressed when Al-exposed cells modify their metabolic functions to assemble metallothioneins and phytochelatins to immobilize Al-ions and detoxify from exposed cells, respectively. Furthermore, it has been established that various Al-toxicity growth studies associate Al-resistance with metallothioneins and phytochelatins in metal-exposed plants with metal-tolerance. Extracellular resistance involves cellular hardening of root cell surfaces exposed to Al-ions to reduce Al affinity to be absorbed through the plant roots.

2.4 The role of *Bacillus subtilis* in plant abiotic stress

B. subtilis is well known for its eco-friendly properties in crop protection, especially its potential to ameliorate soil chemical milieu to threshold suitable for plant root growth. This bacterium is a rod-shaped, gram-positive, catalase-positive facultative anaerobe with the ability to adapt to stressful soil conditions (Kempf and Bremer, 1998). However, a lot of research emphasis was focused on determining its role in suppressing both soil borne and airborne pathogens of economic importance, with the result of less interest on its potency to ward-off abiotic stress in plants. Though there are limited studies conducted to determine biological functions of bacteria in plants growing under abiotic stress, in particular heat-stress and aluminium, *Bacillus* bacteria have been solely shown to synergistically work in concert with plants to enable sustained life of floral and faunal systems both in edaphic and terrestrial eco-cosmisms (Moore and Helmann, 2005). This bacterium is one of the first microorganisms to be applied in agriculture. As a result, the bacterium is a premium component of a number of commercial biological products recommended in crop production.

Effective Microbes (EM) Technology^{TM} microbial stock is made of growth promoting bacteria: *B. subtilis*, *Bifidobacterium animalis*, *B. bifidum*, *B.longum*, *Lactobacillus*

acidophilus, *L. buchneri*, *L. bulgaricus*, *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. plantarum*, *Lactococcus diacetylactis*, *L. lactis*, *Rhodopseudomonas palustris*, *R. sphaeroides*, *Saccharomyces cerevisiae* and *Streptococcus thermophiles* (Higa, 1996; Higa and Parr, 1994; Higa and Wididana, 1991). Although the technology is widely used by farmers in the South Africa as soil conditioner suppressing soil-borne fungal and nematode diseases (Kyan, 1996), limited agronomic and laboratory data have not been generated on its role in bioremediation of soils with low pH, aluminium toxicity and other metal toxicities. With booming farmer evidence about EM products it is important to conduct research trials to substantiate whatever the remarks.

Bacterial communities exposed to metal-toxicities showed different physiological traits and bacterial species composition, as well as development of tolerance to metals (Batool et al., 2014). In general bacteria are characterized by negatively charged carboxyl, hydroxyl and phosphoryl-anionic cell walls attracting any of the highly ionic metals from the soil environment (Moore and Helmann, 2005; Rajendran et al., 2003). In Boyanov et al. (2003) bacterial absorption of metals was coupled with increased metabolic energy expenditures in association with enzyme catalysis, nutrient transport, protein structures and neutralization of ionic charges; which declares that metal-toxins induce changes in bacterial metabolic energy when exposed to high concentrations of metals. These exudates are composed of organic acid anions, inorganic anions (HCO_3^-, OH^-, H^+), phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides and gaseous molecules (CO_2, H_2) (Dakora and Phillips, 2002), which in one way or the other amounts to reduction of soil pH and lower oxidative state of metal-toxins. This explanation declares that remediation of soil`s metal-toxins is a biochemical strategy adjudicated through natural dynamics. In that case a detailed characterization of metal-tolerant bacterial strains is necessary. In reducing environments, FeRB and sulfate-reducing microorganisms can immobilize some metals like Cr and U by direct enzymatic reduction to an immobile and less toxic form (Lovley et al., 1998; Ganesh et al. 1997). This shows that *Bacillus* has regenerative properties associated with changing soil conditions including changing reduction of soil metal oxidation states. In Weilharter et al. (2011) and Batool et al. (2014) different metal-exposed bacterial communities exhibited different physiological traits and changes in species composition.

CHAPTER 3

3. OMICS TECHNOLOGIES CAN IMPROVE OUR UNDERSTANDING OF THE INFLUENCE OF THE ENVIRONMENT ON THE GROWTH AND DEVELOPMENT OF OKRA – A REVIEW (submitted for publication)

3.1 Abstract

Recently, omics technologies have advanced into an epitome for discovery of novel genes, proteins and metabolites with conventions discrediting traditional studies which were popular for evaluating plant physiology. Out of these traditional treatises most observations relied on easy-to-observe plant morpho-anatomical traits and low-throughput biological responses of a single or a few molecules. In the advent of omics technologies it is possible to study multiple responses involving a multitude of molecules. Leading crops like maize, rice and wheat were the first to be studied using omics technologies and the less significant crops are only following. An example of such a less significant crop is okra which has not been extensively studied using omics technologies. Just as it was the case with leading crops, attention should now focus on a promising crop like okra for omics studies. As we argue this point, we limit our discussion to the influence of heat-stress, aluminium toxicity and exposure of the germinating okra seed to *Bacillus subtilis*.

Keywords: Okra, heat-stress, aluminium-toxicity, *Bacillus subtilis*, omics technologies.

3.2 Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] is an annual, warm-season, erectophile and multipurpose crop greatly valued for its vegetable pods, seed oil enriched with lysine and tryptophan amino acids, and paper pulp fibre (Moniruzzaman et al., 2007; Muoneke and Mbah, 2007). This crop is adaptable to high temperature regions with marginal soils (Guddadamath et al., 2011). As a widely consumed vegetable with significant nutrition amongst low-income households in poor agrarian countries okra warrants attention from the research community. South Africa as a country with

much less ideal environmental conditions; requires many alternative crops which are more adaptable to less favourable conditions. For instance, the very populous and vast provinces of Limpopo, Mpumalanga and KwaZulu-Natal are the driest (Archer and Tadross, 2009; Archer et al., 2008; Kruger and Shongwe, 2004) which over and above this harsh climate also experience unpredictable heat-stress episodes which naturally occur with protracted periods of drought compounding the consequences of other abiotic stresses like aluminium toxicity. In these regions it is worthwhile to constantly search for alternative crops especially with the looming threat of climate change. Okra is one such crop which can be a suitable alternative. Records show that South African soils have an average pH ranging between 3.5 and 4.5; which therefore exacerbates plant exposure to high concentration of aluminium (Malcolm and Andrew, 2003; Sumner and Noble, 2003). Soil aluminium-toxicities jeopardise, not only root development in crop plants, but also reduce soil microbial diversity (Litchfield and Gillevet, 2002). Singularly, heat shock and metal-toxicities have been determined to cause significant perturbations in plant physiology and soil microbial dynamics (Abate et al., 2013; Najemnikova et al., 2007; Wardlaw et al., 2002). Given the less favourable physical conditions of South African cropping systems, okra therefore remains a potential crop which may be adapted to the changing climate and marginal to poor soils. Because okra has not been extensively studied, it becomes difficult to predict its success as a new crop in South Africa. As a promising beneficial bacterium we also suggest that there should be studies on the effects of *Bacillus subtilis* on the growth and development of okra. With omics technologies, knowledge on okra, especially, the influence of heat-stress, aluminium toxicity and exposure of the germinating seed to *B. subtilis* can be rapidly gained. The purpose of this review was to demonstrate that omics technologies can help us to expand our knowledge of okra rapidly. Firstly, we will show that many aspects of the influence of heat-stress, aluminium toxicity and *B. subtilis* on okra are unknown. Secondly, we will demonstrate the tremendous benefits of omics technologies in established crops, and therefore indicate that similar achievements can be extended to okra.

3.3 Historical perspective on the need to apply omics technologies to study okra

In the earliest study or observation of plant growth, a Belgian physician, Jan van Helmont, concluded that in the plant environment, water was responsible for the

growth of the plant. Since that study which was conducted in the seventeenth century, more evidence highlighted that the growth and development of the plant is dependent on its environment. Studies in the 18th century showed that light and carbon dioxide were essential in the growth of plants. More substance to studies on the growth of plants was added by cellular and molecular biologists in the twentieth century. These workers studied chemical interactions between plants and their environment. To reach easy conclusions on plant processes it was convenient to study plants that were easy to grow and the need to produce food also informed the decision to study important crops. Most breeding efforts were on wheat and rice, culminating in the Green Revolution of the 1960s. Genome sequencing was adverted and genomes of "model plants" would be studied first and others later. The weed *Arabidopsis* and important crops like rice became model plants and received more attention than any other plant. Plants like okra were left desolate. Because of the rapidly changing climate, there is a need to look at potential alternative crops to afford them the same attention that was paid to convenient and important food crops. This review is inspired by the fact that the high-throughput, rapid and reliable nature of omics technologies will easily level the playing fields. As such, orphan and less important crops like okra will stand to benefit. For the purposes of this review, we focus on the influence of temperature, aluminium toxicity and exposure of the germinating okra seed to *B. subtilis*.

3.4 Temperature

To date, modern treatises have established that environmental temperature induces state changes in physical and chemical processes of plants. This is dated back from the ancient days through which man’s curiosity to measure the degree of coldness and hotness culminated in empirical universal temperature scales, some of which are still used today. With modern thermometers able to determine the degree of ambient temperature, a lot of studies have been concerned with understanding the association of changing climate and crop responses. From most of temperature growth-studies it is evident that plant physiological responses are assessed based on the causative observation between habituation and distribution. This observation served as the foundation for ecosystems classification in which minimum and

maximum temperature levels were used to discriminate regions of cold- and heat-shocks on a global scale. To a great extent these studies could not warrant distinctive treatise on the effects of temperature variation on the biochemical and physiological functions in plant growth and development. As such it is misleading to rely on the globalised classification which categorises crops into their region of habituation, *viz*; tropical, subtropical or temperate regions (Hopkins and Hüner, 2009), since it overrides and lumps a lot of plant responses.

Traditional studies whose aim was to determine the effects of temperature on plant growth focused on determining such traits as stomatal conductance, chlorophyll content and one or few heat response proteins. This extended to studying biochemical pathways that are responsive to heat stress and further associating them with morpho-anatomical and phenological changes in plants. As if to undermine all previous efforts, with their high-throughput nature omics technologies generate massive datasets to answer at once questions that lingered for years in the low-throughput world. In many leading plants, multiple responses to heat by the plant are known. Besides the excess of other approaches suggesting indices that integrate temperature records with physiological and phenological crop data into robust algorithms, omics technologies stand in a better place than suggested indices to profile multi-functional plant responses to environmental temperature. Adoption of these technologies stands to benefit okra in determining its responses to heat stress and rapid improvement than using traditional indices. This is critical for a plant that may be an important crop of the future.

3.5 Aluminium toxicity

With many established crops such as maize, wheat and cotton having been refined into cultigens it is expected that they will not be able to thrive in soils with high concentrations of metals when compared to hardy crops such as okra. Aluminium is a ubiquitous metal, the third element after silicon and oxygen in the lithosphere; contributing about 7% by mass of the earth's crust and highly associated with soil acidity (Delhaize and Ryan, 1995). With global and sub-Saharan soils dominantly acidic this metal exhibits exceptional phytotoxicity than any of the metals (Abate et al., 2013; Malcolm and Andrew, 2003; Sumner and Noble, 2003). Since aluminium

toxicity may worsen with the changing climate, it is imperative to assess the aluminium resistance of crops which may be more important in the future. Okra is one such crop which must be studied to make it "ready" for the future. Because of omics technologies, information which took many years to generate for the presently leading crops may be generated rapidly in okra.

3.6 *Bacillus subtilis*

Soil microbial diversity constitutes regenerative microorganisms which are treasured in dynamic systems of soil management. These microorganisms synergistically work in concert with plants to enable sustained life of floral and faunal systems both in edaphic and terrestrial eco-cosmisms (Hopkins and Hüner, 2009). In the past these interactions have drawn attention worldwide with special interests to understand their function and role in cropping systems. As a result, unravelling this parable resulted into enormous data showing that soil fungal and bacterial populations play a significant role in suppressing soil-borne pathogens (Agrios, 2005; Salisbury and Ross, 1991). Fungi and bacteria as the prominent inhabitants of the soil have to assert themselves to competitively grow under metal-toxin gradients. In Wenling et al. (2005), *Bacillus* was found to exude Al-chelating compounds which desorb Cs from illite silicate. This supports studies which documented exudate-induced weathering of phyllosilicate mineral weathering from plants and rhizospheric organisms (Robert and Berthelin, 1986; Staunton and Levacic, 1999). Exudates have long been found to provide important reception in the relationship between soil microbes and plant roots, and hence establishing beneficiation interaction. Plant-based exudates act as signals to influence the ability of microbial strains to colonise the roots and to survive in the rhizosphere (Press and Phoenix, 2005); which ultimately sets that there must have a role to play in the remediation of soil metal-toxins.

A diversity of *Bacillus* and *Paenibacillus* populations where found to be copiously associated with the plant rhizosphere (Priest, 1993). This is consistent with the view that root exudates serve as elicitation cues for bacteria. Furthermore, exudates induce bacterial chemostasis towards the rhizosphere and encourage diverse colony interactions. In this behaviour some bacteria coexist with fungal populations, for

example Toro et al. (1997) showed that when *Bacillus* is co-inoculated with *Glomus intraradices* it solubilises phosphates and synergistically increases plant nitrogen and phosphate accumulation. *B. subtilis* has been mostly studied as a toxin producer for the inhibition of fungal and nematode plant pathogens (Leifert et al., 1995).

With the looming events of climate change it is important to identify eco-friendly agents that may ameliorate effects of environmental stress, and more importantly boost plant growth and yield. In that imaginary environment, bacteria like *B. subtilis* may form the bulk of the potential products, especially judging from its significance in plant immunity against economic pathogens. The role of *B. subtilis* in plant growth has long been an area of interest especially with the advent of biological agriculture. *Bacillus* accounts to a cosmopolitan dominance within rhizospheric environments of established and neglected crops. Although it is known that some established crops benefit from *B. subtilis* it remains a challenge to explain the role of the bacteria in the biochemical and physiological functions of plants particularly in response to environmental stress. Although not extensively studied, the response of the plant to treatment with *B. subtilis* is critical, especially in combination with the stress factors which may worsen with climate change, for example temperature and aluminium toxicity.

3.7 Undiscovered okra traits: Responses of genes, proteins and metabolites to heat-stress, aluminium and exposure to *Bacillus subtilis*

Without doubt it will be inexplicable to neglect any potential leverage provided by the omics technologies to generate certainty for the commercialisation of okra in resource-poor agriculture especially given its high harvestable yield volume within a single cultivation season. Also, the crop warrants better nutritional and pharmaceutical value when compared to most conventional vegetables. For example, according to Gebhardt et al. (2004) cooked okra pods boast with 11 mg of vitamin C more than in beet (3 mg; cooked), carrot (4 mg; cooked and raw), vegetable corn (3–5 mg; cooked and raw), cucumber (2 mg; raw), green beans (6–9 mg; cooked and raw), potato (6–8 mg; boiled and fried), pumpkin (5–6 mg; cooked and raw) and spinach (8–9 mg; cooked and raw). In Gopalan et al. (2007) and Varmudy (2011) fresh okra pods were found to have 59 mg of vitamin C, 532 mg of

calcium, 70 mg of phosphorus and 0,7 mg of iron. Okra has better cholesterol-lowering effects than soya protein and almonds (Jenkins et al., 2005) and a high of 3968 [(µmol ascorbate equivalent)/100g fresh weight] antioxidant activity than in spinach (3195) and pea (675, 8). Despite this evidence to support the worthiness of this crop, information on its physiology is scanty. Presently, no high-throughput transcriptomic profiling has been done to identify genes that respond to heat stress and aluminium-toxicity in okra. With high-throughput transcriptome sequencing platforms, information on multigenic responses of okra to heat stress, aluminium and exposure of *B. subtilis* can be generated. Against this backdrop, maize and wheat have greatly benefited from transciptomic studies to investigating the influence of heat and aluminium on plant responses. Similarly, a wide array of genes inducible by heat stress can be identified in okra. Also noticed during our literature search is the lack of studies on proteomics and metabolomics to uncover the responses of okra to heat stress. These kinds of studies are well documented in the more established crops.

3.7.1 Heat-stress

Global studies of gene, protein and metabolite expressions have benefited the improvement of maize, wheat and cotton. In these crops studies uncovered that genomic expression of protein functions and metabolic pathways helps in elucidating to a great extent mechanisms of plant heat-tolerance which are associated with yield stability. No such documentation is known in okra although it is serving as a primary nutritional source in marginal to poor agrarian regions. For example, a genome-wide analysis in maize grown at 42°C revealed 71 genes, with 25 up-regulated representative heat-shock factor (Hsf) genes annotated as *Zea mays* heat-shock factors (*ZmHsfs- 01–25*) and their respective protein domains (Lin et al., 2011). These genes were further classified into major classes (class A, B and C) according to their structural characteristics and phylogenetic orthologs with maize, *Arabidopsis* and rice; and the class A genes were further divided into 10 sub-classes. The results fascinatingly unravelled the possibility of the maize genome to have experienced heat-shock factor gene losses during the course of its evolution and genetic improvement. In this regard, although the maize genome remains a global model

plant for genetics and evolutionary research, the results substantiate the need to characterise neglected crops like okra in order to accelerate discoveries of novel genes. Beside the heat-shock factor genes, a broader comparative analysis of Chinese Spring and TAM107 wheat genotypes expressed other genes coding for the production of heat-shock proteins, transcription factors and proteins responsible for the synthesis of a wide variety of metabolites, calcium and sugar signal pathways, RNA metabolism, and ribosomal proteins, as well as proteins related to other abiotic and biotic stresses (Qin et al., 2008). With okra accustomed to environments prone to gradual temperature increases of more than normal thresholds it is expected that the crop harbours novel heat-responsive genes that make the crop sustainable in climate changing environments.

The stability of heat-tolerant genomes is explained through the expression of heat-shock transcription factors that regulate heat-responsive genes (Hartl and Hayer-Hartl, 2002). Heat-responsive genes control translation and therefore expression of heat-shock proteins. In the model plant, *Arabidopsis*, heat-shock proteins and transcription factors were characterised (Agarwal et al., 2001) and it was established that plant heat response is mediated by Hsp70 proteins (Richter et al., 2001), 7 Hsp90 proteins (Bukau et al., 2006), and up to 8 Hsp100 protein family (Frydman, 2001) and others. These proteins are renowned for their ability to protect cells against severe heat-induced dehydration and therefore maintaining functions in protein folding, intracellular distribution and degradation of other developmental proteins (Harlt and Hayer-Harlt, 2002). One of their roles is to uphold cellular membrane integrity with normal state that limits cellular injury. While on the other hand failure to do so amounts to altered enzymatic activities of proteins, in particular heat-shock proteins, responsible for the expression of metabolome that plays a critical role in correcting homeostatic imbalances induced by heat-stress. Though only few high-throughput non-targeted metabolomics studies have been conducted on plant response to heat stress it has been proved that a diversity of secondary metabolites are expressed during acclimation to heat-stress; some of which are responsible for inducing thermotolerance. In concurrence with traditional studies, but in this case widely elucidating further more biological functions, metabolomics with Arabidopsis studies showed that heat-stress compels plants into excessive respiration and therefore expressing an array of metabolites, amongst them

intermediates of tricarboxylic pathway, scavengers of free radical, stabilizers of cellular membranes and compatible osmolytes. Against the back drop of treasurable achievements in using omics technologies it will be important to conduct such studies with okra in order to improve its status to the level of conventional crops. Furthermore, comparing okra with conventional vegetables such as tomato and potato a great deal of progress has been established to develop genotypes which thrive under heat-stress conditions. One such account is the Israeli cherry tomato and potato cultivars which amounted to a successful vegetables industry in the country. With okra widely distributed in a wide array of regions including Africa it is possible to discover landrace genomes conserved with heat-tolerance.

3.7.2 Aluminium

Biochemical pathways and transcriptional regulation of aluminium tolerance genes have been studied in wheat and maize while such accounts are lacking in okra. In most of the omics studies the focus is to determine the comparative expression of known genes from model plants, in this regard Arabidopsis, wheat and maize, into a selected less studied crops such as okra. The primary goal is to unravel genes, proteins and metabolites playing critical roles in off-setting aluminium phytotoxicity. Genes broadly classified into aluminium-activated malate transporter (ALMT) family were the first to be found enhancing expression of aluminium tolerance in wheat (TaALMT1) (Sasaki et al., 2004), Rye (ScALMT gene cluster) (Collins et al., 2008), Arabidopsis (AtALMT1) (Hoekenga et al., 2006), and *Brassica napus* (BnALMT1 and BnALMT2) (Ligaba et al., 2006). Others are those grouped in gene family, multi-drug and toxic compound exudations (MATE), including among others SbMATE1 in sorghum (Magalhaes et al., 2007), HvAACT1 in barley (Furukawa et al., 2007a,b), ZmMATE1 in maize (Maron et al., 2010), TaMATE1 in wheat (Ryan et al., 2009), AtMATE in Arabidopsis (Liu et al., 2009), and ScMATE2 in rye (Yokosho et al., 2010/1). Beside that these genes are equally expressed in both monocotyledonous and dicotyledonous crops growing in acid soils characterization of more genes in the majority of neglected crops is still to follow. These gene families are further characterized by the exudation of specific organic anions. In plants expressing genes from the ALMT family malate anion is exuded and as for MATE genes citrate. These

exudates were much reckoned by researchers after they were further related to specific transporter proteins and genes as well. This relationship stifled discoveries in this regard to be only focused on physiological and molecular responses that explain root exudation mechanisms. However, with the high-throughput omics technologies genes including those within and out of the root exudation spectrum can be discovered to comprehensively explain aluminium tolerance better. With okra having a wide distribution in regions with poor soils it is expected that it will exhibit tolerance to high aluminium levels.

3.7.3 Exposure to *Bacilus subtilis*

B. subtilis is a plant growth stimulant and biocontrol agent against major fungal disease. As a bacterium *B. subtilis* has been studied and also its associations with plants have been documented. However, few omics studies have been conducted to reveal plant responses induced by *B. subtilis*, including also its role in plant heat- and -aluminium tolerance. This scantity of omics studies to determine the response of the plant to exposure to *B. subtilis* is even more pronounced in okra. Because *B. subtilis* can stimulate the growth of okra, it is important to determine the genetic basis of their interaction. As high-throughput, rapid and reliable methods, omics technologies can help us achieve these objectives. Despite extensive accounts which reflect the bacterial role in crop protection a lot of scepticism remains to elaborate the role of *B. subtilis* in plant responses to abiotic stress, more in particular heat-stress and aluminium. From the perspective that plant root exudates serve as elicitation cues to specific soil microbes it is expected that this relationship can explain the genetic basis of plant response to *B. subtilis* exposure. Unfortunately, majority of studies on plant-bacteria interactions focused more on the role of lipopeptides from the surfactin, iturin and fengycin families (Bonmatin et al., 2003; Peypoux et al., 1999) and other factors which cause induced systemic resistance (ISR) in plants infected with pathogens. With high-throughput omics technologies genes, proteins and metabolites which are receptive to *B. subtilis* can be determined, and aid in determining okra responses when exposed to the bacteria.

3.8 Importance of these studies in okra

To date, growth studies in heat-tolerance and aluminium-tolerance with any of the commercial cultivars and selected okra landrace populations are limited. And such studies are much sought after in developing stress-tolerant genotypes suitable to various cropping systems. Reviewed studies helped to draw inconsistencies in plant responses to stress, and in particular discerning which evaluation methods will be reliable and cost effective. Okra is widely cultivated in regions with unpredictable peak temperatures, drought, and metal-polluted acid soils. With information highlighting superiority of *Abelmoschus esculentum* from *A. caillei* germplasm in such conditions; it will be important to integrate their gene factors in order to develop cultivars adaptable to harsh environmental conditions. Recently, transcriptomics of different okra populations has been characterized which helped to identify okra gene sequence for the first time (Schafleitner et al., 2013). It will be important to further apply these omics technologies in determining heat-stress tolerance, aluminium tolerance and the response of the germinating okra seed exposure to *B. subtilis*.

Omics technologies have the potential to generate high-throughput information through which responses of okra to heat-stress, aluminium-toxicity and exposure of the germinating okra seed to *B. subtilis* can be comprehensively understood. These advances will provide certainty for the adoption of okra in resource-poor farming regions such as those of South Africa.

CHAPTER 4

4. INLUENCE OF HEAT, ALUMINIUM TOXICITY AND EXPOSURE TO *BACILLUS SUBTILIS* ON THE GERMINATION OF OKRA (submitted for publication in a journal)

4.1 Abstract

In the looming threat of the consequences of global warming okra is an alternative crop because of its hardy features. Despite its promise, the germination physiology in particular influences of heat, aluminium concentration and seed exposure to *Bacillus subtilis* of okra have not been extensively studied. The aim of this study was therefore to investigate the influence of heat, aluminium concentration of the germination medium and exposure to *B. subtilis* on the germination responses of okra. A germination factorial experiment was conducted using Petri dish assays and germination and coleoptile data was captured from the different treatments. Quantities of cations, anions, carbon, nitrogen and secondary metabolites were determined. Experimental data was analysed and inferences from that analysis are presented.

Keywords: Aluminium toxicity, *Bacillus subtilis* metal bioremediation, ionome, metabolome, *Abelmoschus esculentum*, germination physiology.

4.2 Introduction

The biology of seed germination spawn wonders that are habitually missed in the hinterlands of conventional plant improvement. This is as a result of the minuscule nature of seed structures which in most cases lead to germination assays less accountable to biochemical homeostatic dynamics. Homeostasis is the capacity through which genomes resist extreme changes in physiological mechanisms owing to perturbations in growth stimuli. In some studies this phenomenon has been explained through stoichiometry of ionomic relationship. In order to apply similar approach in seed germination physiology it will be rather helpful to consider showing inconsistencies of popular hypotheses in germination studies. These studies have at

most popularized the view, in this regard referred to as canonical hypothesis; which insist that instigation of elementary growth in the accumulation of both ions and metabolites during seed germination is exclusively dependent on photosynthesis of primary leaves. In contrast to this hypothesis, Oloyo (2004) showed that germination of *Cajanus cajan* seed is positively correlated with increased accumulation of fat, crude fibre, total ash, acid-insoluble ash, cell wall carbohydrate, hemicellulose, iron, manganese, calcium, magnesium, copper and phosphorus. Through these results there is clear evidence that shift in ionomic homeostasis occurs even before the development of primary photosynthetic machinery. In this regard, it is rather important to consider seed ionomic changes to be acquiescent with germination environment. Hardier seeds such as okra are structurally bound to overshadow anti-germination effects (Demir, 1997; Egley and Elmore, 1987). These seeds are deemed dormant because of their inability to flush dormancy-specific metabolites such as cytokinin, abscisic acid, unsaturated lactones, phenolic compounds and cyanogenic compounds, and therefore switching-off genes controlling physiological transition from dormant to germination state (Holdsworth et al., 2008). In Oloyo (2004), germination progression was proved to be in phase with the reduction in total oxalate and phytic acid and augmented levels of tannins, phenolics and trypsin activation. This antagonistic relationship further confirms that germination physiology is the flip side of dormancy seed state, as also is reflected in the regulation dormancy-positive genes such as ABA1 (abscisic acid1; Bentsink et al., 2006) deficient1, ABA3,4,5 (abscisic acid insensitive 3, 4, 5; Liu et al., 2007; Piskurewicz et al., 2008; Zheng et al., 2012), DOG1 (delay of germination1; Bentsink et al., 2006; Nakabayashi et al., 2012) and dormancy-negative genes such as ACO1,4,5 (1-aminocyclopropane-1-carboxylate oxidase 1, 4, 5; Wang et al., 2013). These responses substantiate that environmental condtions can reverse germination, if not relax metabolic progression.

In as much as hydration is critical to instigate coordinative metabolism between embryo and nutritive layer of seeds, temporal adjustments of electrochemical gradient and ion movement are equally important. This dynamic is crucial to validate optimum homeostasis in the activation of respiration (Bewley and Black, 1994), restoration of membrane structure (Osborne, 1993), mobilization of energy reserves (Gallardo et al., 2002), reinvention of cell cycle (De Castro et al., 2000; Vasquez-

Ramos and Sanchez, 2004), seed coat rupturing and emergence of coleoptiles (Groot and Karssen, 1987). As a result, unequal distribution of anionic and cationic charges between germination medium and seed supports selective ion flow depending on the concentration gradients. Furthermore, Soeda et al. (2005) showed that osmotic stress has the ability to reverse germination-related genes, confirming that successful germination is as a result of variation in germination environment. Given climate changing conditions it will be important to determine germination physiology of okra under heat stress and aluminium toxicity as well as assess the role a bioremediator like *Bacillus subtilis* can play in relieving okra from heat and aluminium stresses. Rather than relying on canonical hypothesis the study focused on determining ionomic and metabolomics changes as explanatory factors of homeostatic shifts preceding okra germination phenotype in responses to various aluminium concentrations, different temperature levels and exposures to *B. subtilis*. The study relagates the hypothesis on the basis that it is deficient with capabilities which will enable plant stress physiologists to understand the whole system of metabolic pathways taking place during seed germination, in particular establishment and progression of embryonic development.

4.3 Materials and Methods

4.3.1 Plant material and bacterial culture

Okra cultivar, Clemson Spineless, was selected for this study. The seed used for this germination study was taken from the same batch, commercially produced by Starke Ayes. A set of the seed was imbibed in LB plated *B. subtilis*, strain BD233, obtained from the Agricultural Research Council – Plant Protection Research Institute (Republic of South Africa).

4.3.2 Layout of germination experiments

Okra seed germination assays were conducted on moist filter paper in 90mm diameter Petri plates sealed with parafilm. The medium of germination was distilled

water for the control (0 M) and varying concentrations of aluminium chloride ($AlCl_3$), 0.001 M, 0.01 M, 0.05 M and 0.1 M which served as experimental treatments. The effects on germination were those of $AlCl_3$ and not aluminium as it may loosely be mentioned in this manuscript. Each treatment of aluminium chloride concentration was split between seed either coated or uncoated with *B. subtilis*. The seed, 80 in each Petri dish, was incubated in three temperature treatments 22°C, 25°C and 37°C. Treatment levels in both aluminium chloride and temperature treatments were selected based on preliminary results (Figure 4.1 - 2), which showed greater inhibition in okra seed germination from 0.1 M and 1 M aluminium concentrations and 40°C temperature level, respectively. The treatments described aboved were allotted in a 5 x 3 x 2 completely randomized factorial design with five replicates, and the experiment was repeated in three cycles. In total 450 Petri plates were examined in the data collection.

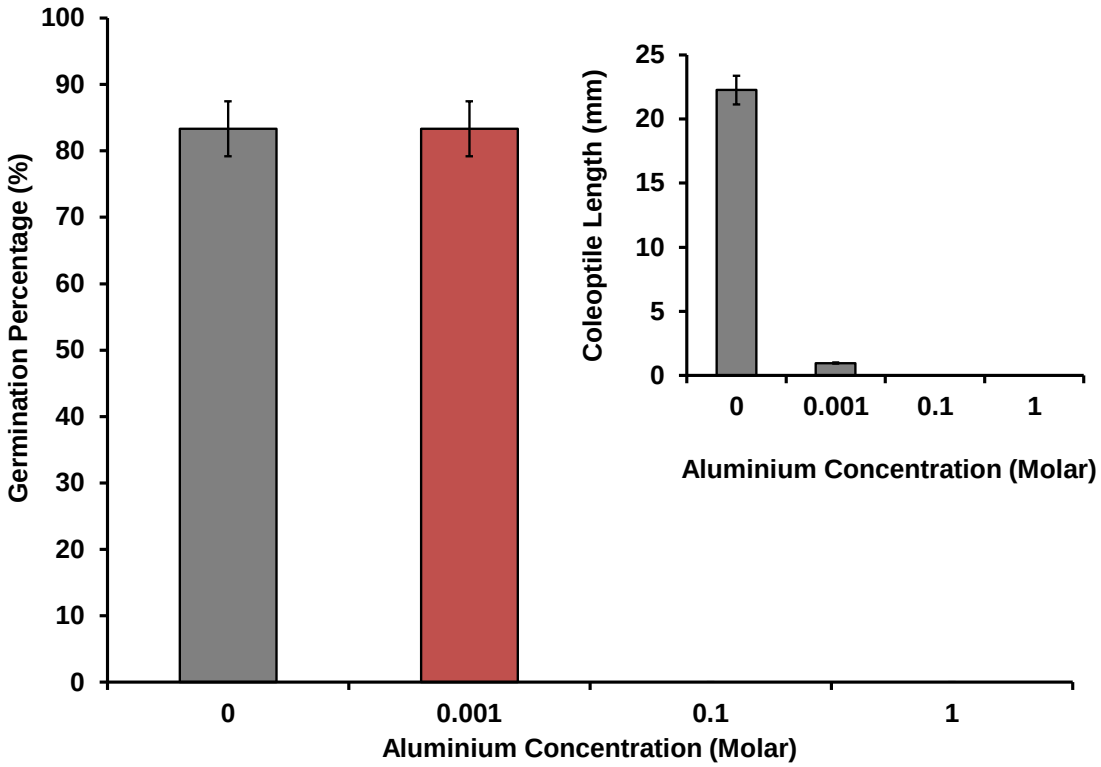

Figure 4.1: Preliminary. Germination percentages and coleoptile lengths of okra (*Abelmoschus esculentum*) seed in response to various concentrations of aluminium.

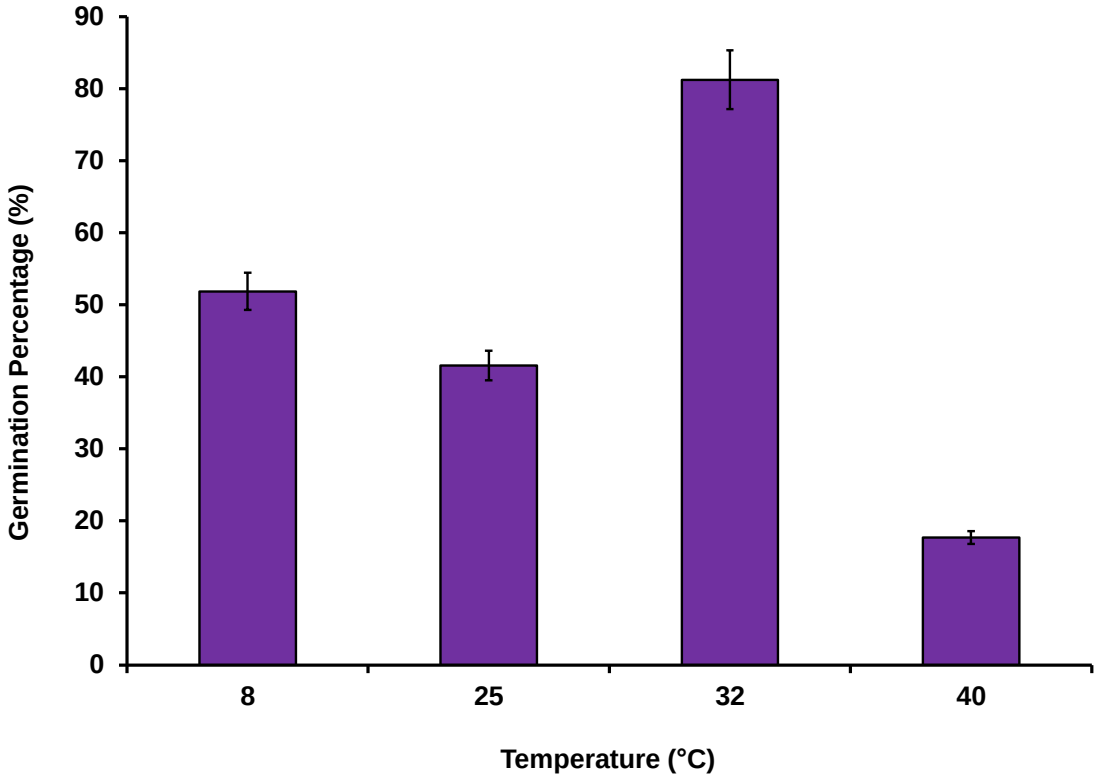

Figure 4.2: Preliminary. Germination percentages of okra (*Abelmoschus esculentum*) seed in response to various temperature levels of the germination environment.

4.3.3 Data collection

4.3.3.1 Phenotype data

Daily scores of germinated seeds were assessed from the second to the fifth day after initiation of germination. During termination, five days after the initiation of the experiment, 10 seeds with the longest coleoptiles had their coleoptiles measured using a digital caliper.

4.3.3.2 Ions and ^{1}H NMR spectra generation

Fifty and hundred (from two replicates of all experimental cycles) germination plates were selected for ionomics and ^{1}H NMR analyses, respectively. Germinated seeds from selected plates were crushed with liquid nitrogen using a mortar and pestle. Finely ground germinated seed were transferred into 2ml Eppendorf tubes and refrigerated at -20°C until analyses.

Inorganic carbon and nitrogen were quantified using CHN analyser, anions (PO_4^{3-}, SO_4^{2-}, NO_2^-, NO_3^-, Cl^- and F^-) with ion chromatography (IC) and cations (Al^{3+}, Fe^{3+}, B^{3+},Cu^{3+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, K^+, Na^+) with inductively coupled plasma optical emission spectrometry (ICP-OES). P content was determined using spectrophotometry after acid digestion of ashed samples. As for ^{1}H NMR analysis, metabolites were extracted with methanol:water (50:50 (v/v)). The mixture was vortexed for three minutes, sonicated for 20 minutes and centrifuged at 18000 rpms for 20 minutes. Remaining solid particles in the supernatant were removed by filtration through a piece of cotton wool. The supernatant which contained secondary metabolites was dispensed into NMR tubes. NMR spectra was generated using a 600 MHz NMR Varian spectrometer.

4.3.4 Data analysis

4.3.4.1 Phenotype responses: Germination percentages

Phenotype data for germination percentages and coleoptile lengths were evaluated for normality using Shapiro-Wilk Normality test, and the data was found to be significant at 1% probability level. The data was then subjected to ANOVA procedure using Statistix version 10. Significant treatments at 5% treatment probability level were partitioned into degrees of freedom, mean sum of squares and F-values to compare the variance contributed by the sources of variation. Means were separated using the Fischer`s Least Significant Difference (LSD) post-test technique at 5% probability level (Gomez and Gomez, 1984). Discretely, significant means (n

= 5) from Experiment 1, 2 and 3 were processed through GraphPrism software to populate bar graphs with error bars.

4.3.4.2 Ionomic concentrations and ^{1}H NMR spectra

Quantities of ions were pooled from aluminium and temperature treatments, and further averaged across bacterial treatment. Furthermore, relative impact [= (*B. subtilis*/Control – 1)] x 100] index was computed using data pooled from aluminium concentrations and temperature treatments; to determine the general quenching effects of bacterial treatment in germinating seed.

Phase and baseline correction of the NMR spectra were done using ACD/NMR Processor. NMR intensities were selected from the range zero to 14 ppm and exported as ASCII files. The data was populated into the Multibase 2014 for principal component analysis, partial least squares regression – discriminant analysis and partial least squares – enhanced discriminant analysis. Through these analyses biplots were generated to discriminate between the various treatments.

4.4 Results

4.4.1 Phenotype response: Germination percentage

4.4.1.1 Factor interactions: Aluminium concentrations (A_C), bacterial seed treatment (B_S) and germination period (G_P)

With respect to germination percentages first- and second-order interactions between aluminium concentrations, *B. subtilis* treatment and germination days were assessed from all temperature environments. In all temperatures, except 37°C, variation contributed by A_C x B_S interaction in germination percentages was consistently greater than the sum of other interaction terms (Table 4.1).

Furthermore, a triad (second-order) interaction of all predictor factors was significant in 22°C and 25°C temperatures, whereas in 37°C was no significant.

4.4.1.2 Aluminium concentration

In general aluminium concentrations had more contribution in variation of germination percentages in all temperatures, however much higher in 22°C and 25°C temperatures, respectively (Table 4.1). In Figure 4.3 – 6, it is evident that 0.001 M concentration invigorates germination percentages at lower temperatures. This concentration threshold for stimulation of germination was widened when temperature increased from 22°C to 25°C, but hampered at 37°C level. The level of aluminium concentration which inhibits okra germination is within the selected range as germination percentage was observed to increase or decreases between 0.001 M and 0.01 M concentration range (Preliminary results: See Figures 4.1 and 4.2). In agreement with Alamgir and Akhter (2009), germination and coleoptile length were stimulated at lower aluminium concentrations. In the study of Hoekenga et al. (2003), 1mM concentration of aluminium was used in the identification and characterization of aluminium tolerance loci in Arabidopsis, Landsberg erecta x Columbia, genotypes. However, in the case of okra the results suggest that the concentration will not be suitable for the assessment of aluminium tolerance. Instead, 0.01 M and 0.05M concentrations which showed substantive reduction of germination percentages and coleoptile lengths of okra seeds without *B. subtilis* coat treatment were suggested.

4.4.1.3 Temperature

This study showed that the okra seed germination differs with the temperature of incubation. At 22°C and 25°C no significant differences in the percentage germination were observed, and a drastic reduction in percentage germination was observed at 37°C in all the three experiments (cycles).

4.4.1.4 *Bacillus subtilis* treatment

In addition to aluminium concentration-specific germination response *B. subtilis* treatment further improved germination percentages. The treatment had most contribution in variations of germination percentages at 22°C and 25°C, while least in 37°C. At 0.01 M *B. subtilis* treatment appears to have remedial/quenching effects on aluminium toxicity because *B. subtilis* coated seed had the highest germination percentages at 0.01 M rather than at 0.001 M as was the case with the uncoated seed at 22°C and 25°C.

Similar trends in germination percentages were observed in all experiments. The inconsistencies encountered in all three experiments were overridden and more importantly normalizing the standard errors and coefficients of variations as compared to those encountered in the experiments separately.

Table 4.1: Pooled (Experiment 1, 2 and 3). Analysis of variance for the cumulative germination percentage (from days two through five) of okra (*Abelmoschus esculentus*) seed, either coated or uncoated with *Bacillus subtilis*, in different levels of aluminium concentration and in different temperatures.

		22°C		25°C		37°C	
Source of variation	DF	MS	F-value[y]	MS	F-value[y]	MS	F-value[y]
Replicate (R)	4	2.6		26.9		28.91	
$Al(NO_3)_3$conc. (A_C)	4	17681.0	721.08**	20619.5	1485.86**	3760.01	461.84**
Bacillus subtilis (B_S)	1	6325.4	257.97**	8597.4	619.54**	550.85	67.66**
Germination days (G_D)	3	7239.9	295.26**	8316.8	599.32**	1074.58	131.99**
A_C x B_S	4	3954.7	161.28**	3830	276**	140.72	17.28**
A_C x G_P	12	505.1	20.6**	574.6	41.41**	181.34	22.27**
B_S x G_P	3	67.7	2.76**	318.9	22.98**	12.95	1.59[ns]
A_C x B_S x G_D	12	94.8	3.86**	112.3	8.09**	4.49	0.55[ns]
Error	156	24.5		13.9		8.14	
		Mean	33.80	**Mean**	36.58	**Mean**	10.51
		CV%	14.65	**CV%**	10.18	**CV%**	27.14

[y]Refers to F-values of source of variation with significant difference at 1% (**), 5% (*) or [ns] (for no significance difference at either 1% or 5%).

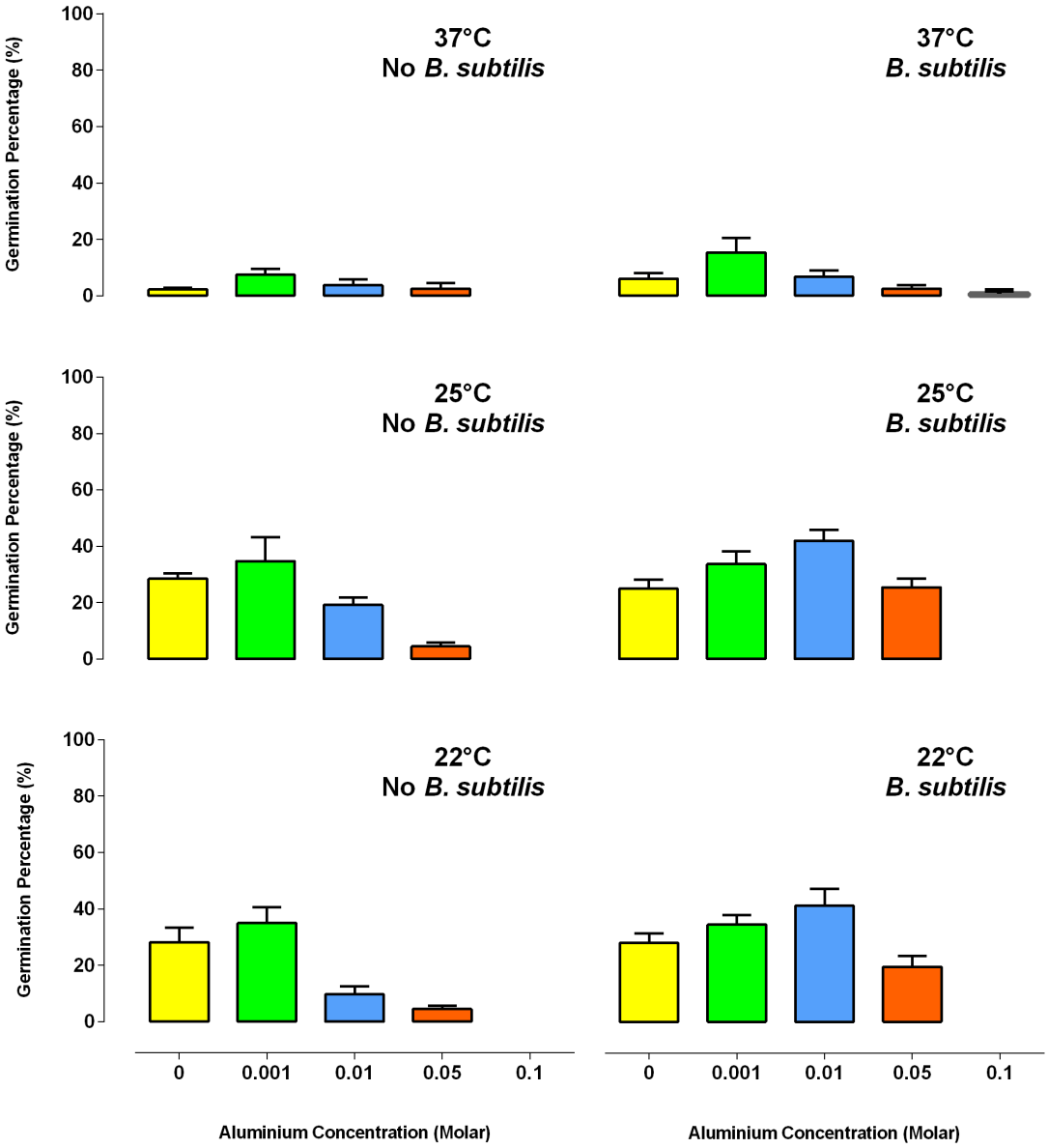

Figure 4.3: Germination of okra (*Abelmoschus esculentus*) seed either coated or uncoated with *Bacillus subtilis* in response to various concentrations of aluminium in different temperatures two days after initiation of germination.

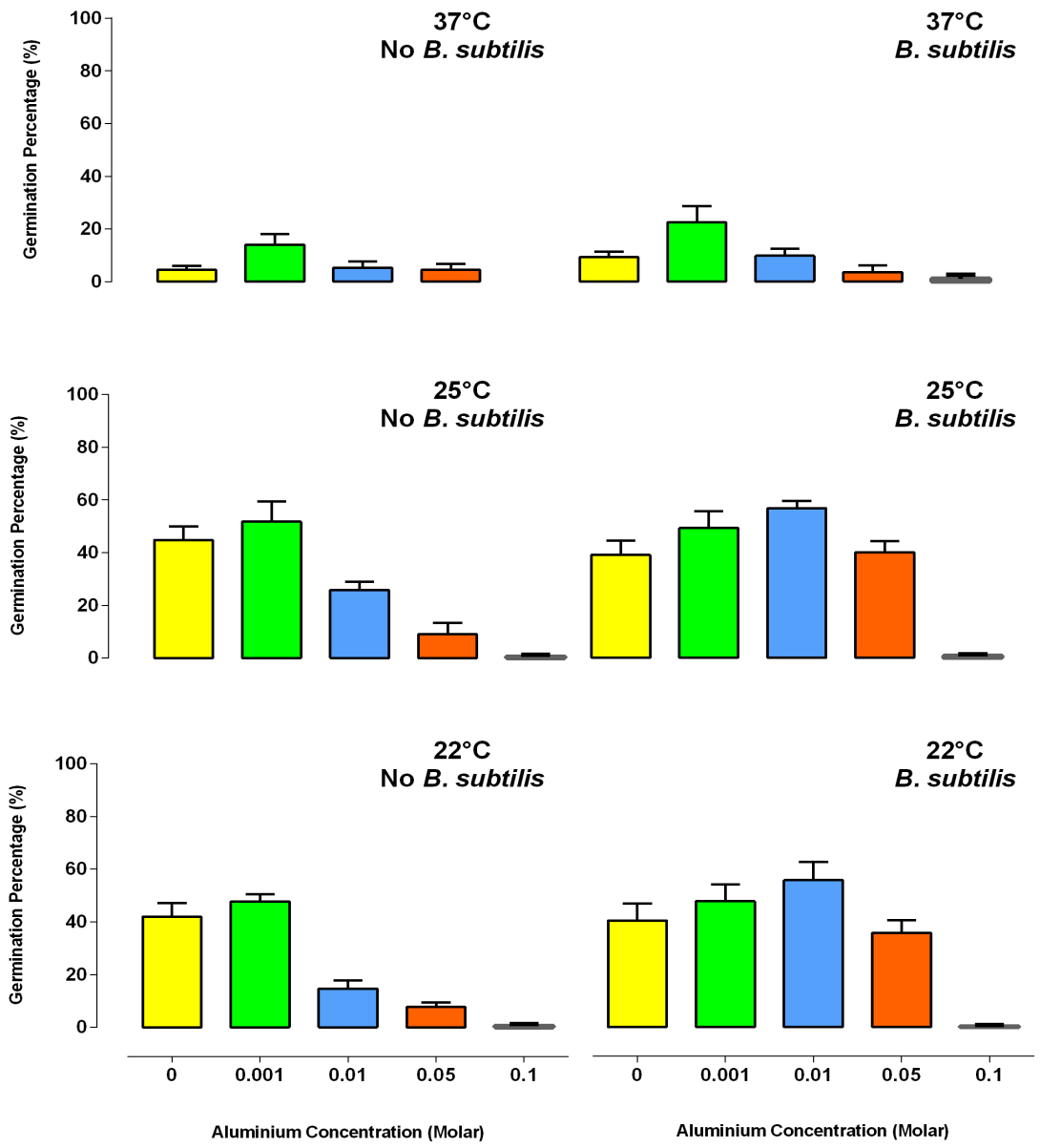

Figure 4.4: Germination of okra (*Abelmoschus esculentus*) seed either coated or uncoated with *Bacillus subtilis* in response to various concentrations of aluminium in different temperatures three days after initiation of germination.

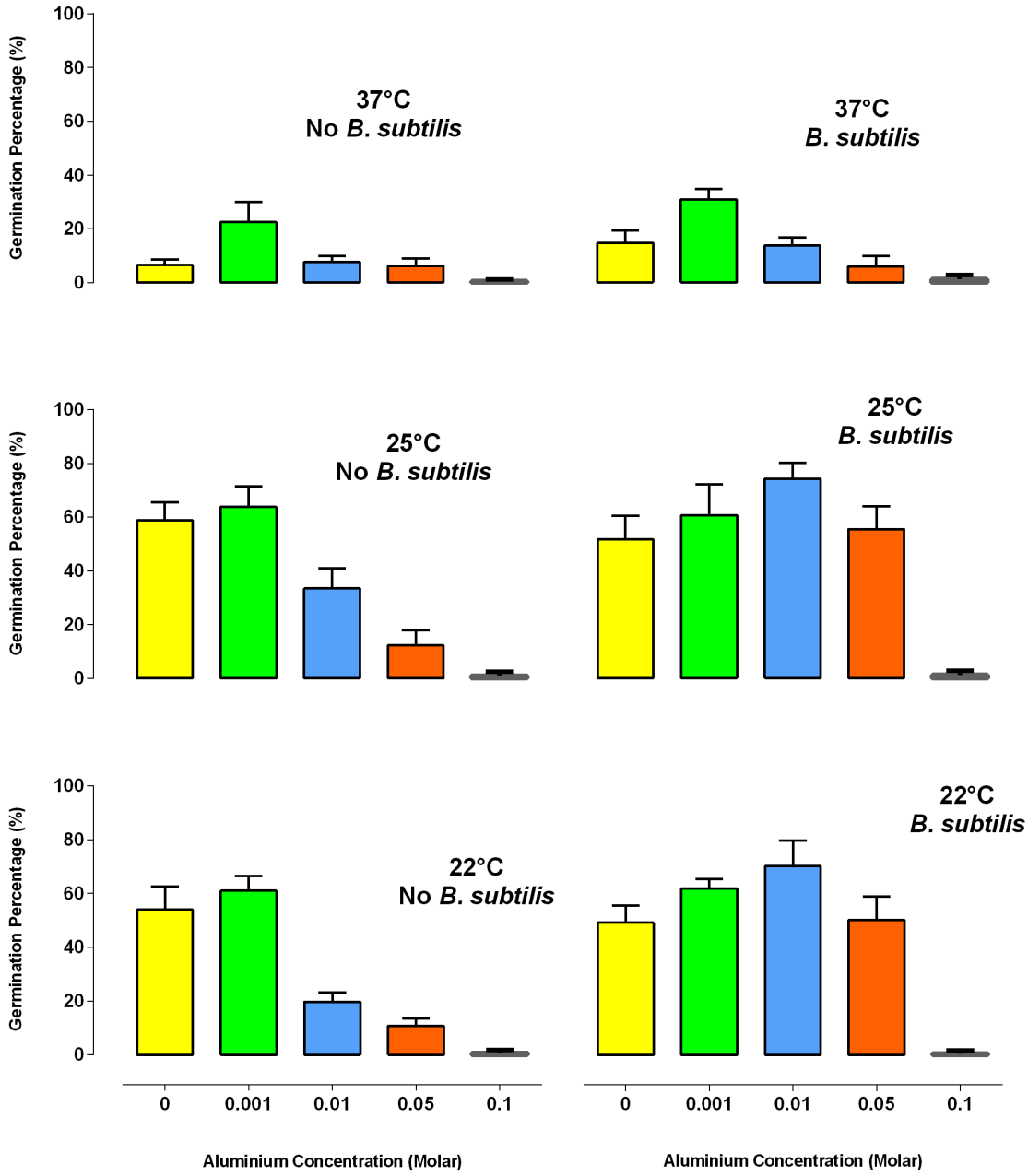

Figure 4.5: Germination of okra (*Abelmoschus esculentus*) seed either coated or uncoated with *Bacillus subtilis* in response to various concentrations of aluminium in different temperatures four days after initiation of germination.

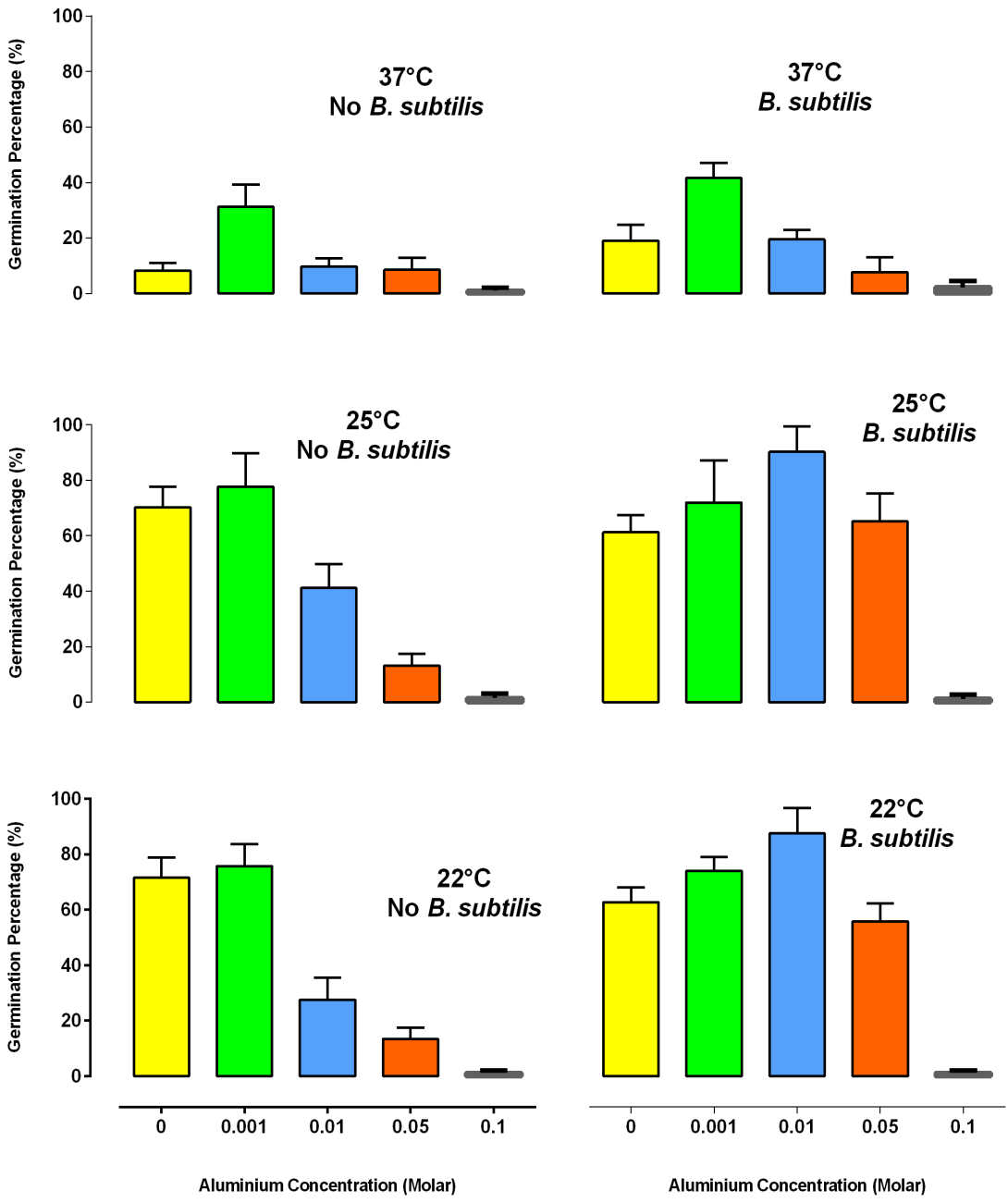

Figure 4.6: Germination of okra (*Abelmoschus esculentus*) seed either coated or uncoated with *Bacillus subtilis* in response to various concentrations of aluminium in different temperatures five days after initiation of germination.

4.4.2 Phenotype response: Coleoptile lengths

4.4.2.1 Factor interaction: Aluminium concentrations (A_C), bacterial seed treatment (B_s) and temperature level (T_L)

The B_sx T_L interaction had greater variance in coleoptile lengths followed by A_c x T_L, A_c x B_s x T_L and A_c x B_s (Table 4.2). Furthermore, 25°C temperature accounted more variance in A_c x B_s interaction than in 22 and 37°C (Table 4.3). The results serve as an affirmation to the view that temperature variation distinguished changes in physiological functions in both germinating seeds and bacterium growth.

4.4.2.2 Aluminium concentration

Similar to germination percentages aluminium treatment had greater contribution in the variation of coleoptile lengths (Table 4.2). This variance was further temperature specific; greater in both 22°C and 25°C germination environments (Table 4.3). The coleoptile grew the fastest in the treatment of 0.001 M aluminum in the absence of *B. subtilis* with almost no growth at all at 0.1 M. This was observed in all the temperatures 22°C, 25°C and 37°C. In the presence of *B. subtilis*, coleoptile growth was the fastest at 0.01 M at 25°C with 22°C greatly differing from 25°C.

4.4.2.3 Temperature

The variance in coleoptile lengths contributed by temperature treatment was high, second after aluminium concentrations (Table 4.2). Furthermore, the effects of other experimental treatments were significantly influenced by temperature environment (Table 4.3). As expected the growth of the coleoptile was affected by temperature levels. At 25°C the coleoptile grew the fastest and the slowest at 37°C (Figure 4.7).

4.4.2.4 *Bacillus subtilis* treatment

In agreement with the germination percentages, bacterial seed treatment had significant quenching effects on coleoptile growth. The variance in coleoptile lengths was 483.2, third after those of aluminium concentrations and heat treatments (Table 4.2). However, bacterial quenching effects were temperature specific. The most quenching effects were observed at 25°C temperature with variance 11 and 15 times greater than variances at 22°C and 37°C temperatures, respectively (Table 4.3).

Bacterial seed treatment was able to widen aluminium concentration threshold from 0.001 M to 0.1 M for coleoptile growth in 25°C.

Table 4.2: Pooled (Experiment 1, 2 and 3). Analysis of variance for coleoptile length of okra (*Abelmoschus esculentus*) seed, either coated or uncoated with *Bacillus subtilis*, in different levels of aluminium concentration and in different temperatures.

Source of variation	DF	MS	F-value[y]
Replicate (R)	4	1.54	
$Al(NO_3)_3$ conc. (A_C)	4	1121.18	944.83**
Bacillus subtilis (B_S)	1	483.2	407.2**
Heat level (H_L)	2	659.54	555.8**
A_C x B_S	4	53.5	45.09**
A_C x H_L	8	81.44	68.63**
B_S x H_L	2	108.46	91.4**
A_C x B_S x H_L	8	75.78	63.86**
Error	116	1.19	
		Mean	7.56
		CV%	14.41

[y]Refers to F-values of source of variation with significant difference at 1% (**), 5% (*) or [ns] (for no significance difference at either 1% or 5%).

Table 4.3: Pooled (Experiment 1, 2 and 3). Analysis of variance for coleoptile length of okra (*Abelmoschus esculentus*) seed, either coated or uncoated with *Bacillus subtilis*, in different levels of aluminium concentration, separated into different temperatures.

		22°C		25°C		37°C	
Source of variation	DF	MS	F-value[y]	MS	F-value[y]	MS	F-value[y]
Replicate (R)	4	0.87		1.39		1.82	
$Al(NO_3)_3$ conc. (A_C)	4	549.6	496.01**	592.6	488.98**	141.86	116.14**
Bacillus subtilis (B_S)	1	51.39	46.38**	610.26	503.56**	38.46	31.48**
A_C x B_S	4	11.49	10.37**	183.83	151.68**	9.75	7.98**
Error	116	1.11		1.21		1.22	
		Mean	8.04	**Mean**	10.93	**Mean**	3.71
		CV%	13.09	CV%	10.07	CV%	29.77

[y]Refers to F-values of source of variation with significant difference at 1% (**), 5% (*) or [ns] (for no significance difference at either 1% or 5%).

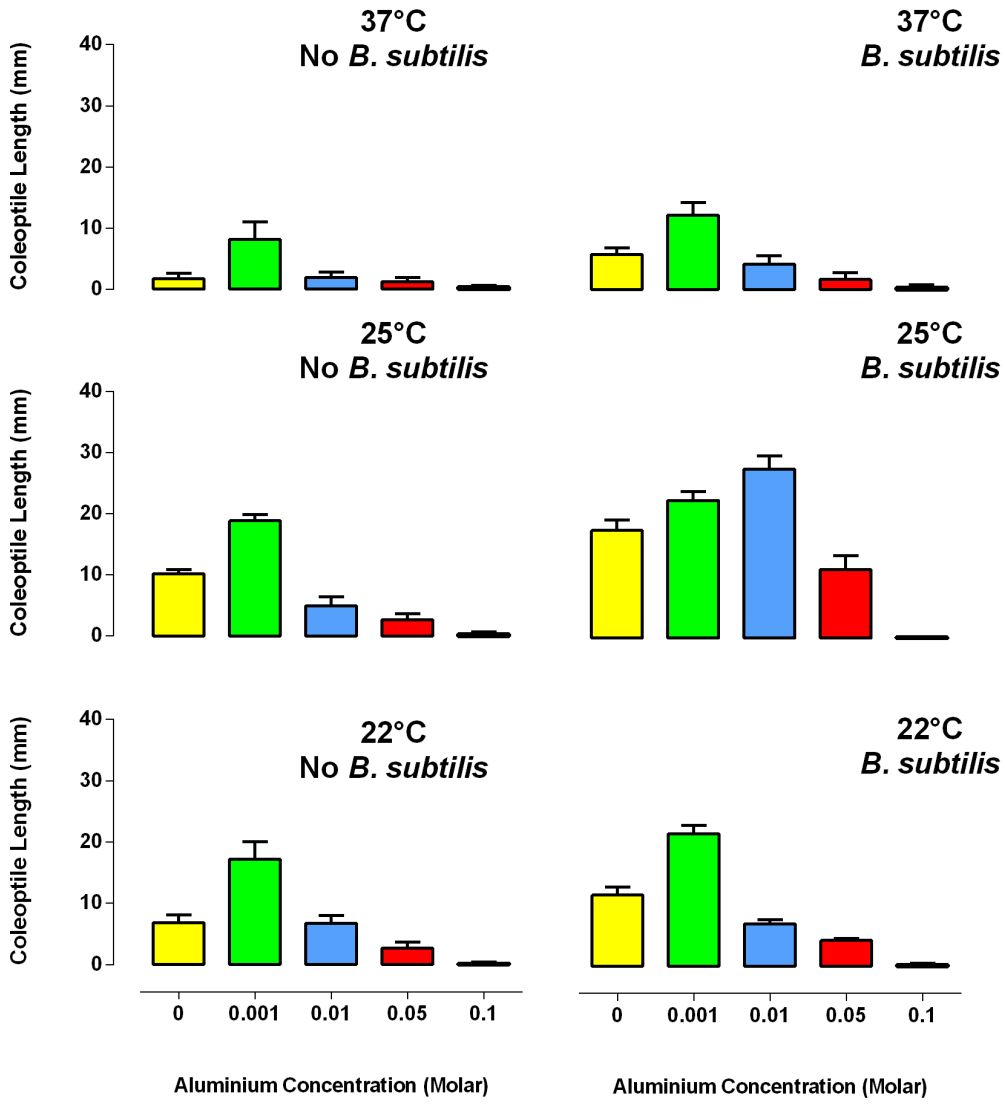

Figure 4.7: Coleoptile length of okra (*Abelmoschus esculentus*) seed, either coated or uncoated with *Bacillus subtilis*, growing in various concentrations of aluminium under different temperatures, measured five days after initiation of germination. Coleoptile lengths represents lengths of 10 longest coleoptiles per treatment, each replicated five times.

4.4.3 Partial ionomics and ^{1}H NMR metabolomics

4.4.3.1 Partial ionomics

Carbon, nitrogen, phosphorus and ions were quantified from finely ground germinated seed of okra. Quantities of the different elements and ions are presented in Table 4.4. Treatments of *B. subtilis* were expected to have elevated quantities of the different elements and ions because bacterial contents contain substances. However, there was no difference between *B. subtilis* treatments and untreated samples indicating that bacterial additions were minute. Because of the insignificant contribution of *B. subtilis* to the total chemical composition of samples, no metabolomic differences were expected between samples treated or untreated with the bacterium.

Table 4.4: Ion concentrations and relative impact of *Bacillus subtilis* treatment in germinated okra seeds. Means pooled from aluminium concentrations and temperature levels.

	Ion	Concentration (mg/kg)		Relative impact (%)[z]
		Control	*B. subtilis*	
Cations	K^+	8196.1	8467.4	3.31
	Na^+	604.3	769.1	27.27
	Mg^{2+}	3355.0	3414.3	1.78
	Ca^{2+}	1437.2	1425.8	- 0.79
	Zn^{2+}	59.9	60.2	0.50
	Mn^{2+}	18.6	18.6	0
	Al^{3+}	3308.4	3143.0	- 4.99
	Fe^{3+}	65.2	70.0	7.36
	B^{3+}	20.9	21.1	0.96
	Cu^{3+}	11.3	11.5	1.77
Anions	Cl^-	2106.9	2166.4	2.82
	F^-	606.2	968.6	59.78
	NO_2^-	16.6	40.4	143.37
	NO_3^-	169.8	131.4	- 22.61
	SO_4^{2-}	629.3	643.8	2.30
	PO_4^{3-}	4172.4	3358.9	- 19.49
Inorganic	C	48.2	48.24	-0.02
	N	4.4	4.1	-5.75
	C/N	11.1	11.8	5.79
	P	5628.8	5703.2	1.32

[z]Relative impact (%) = [(*B. subtilis*/Control – 1)] x 100.

4.4.3.2 ^{1}H NMR metabolomics

The ^{1}H NMR metabolomics was run on the germinated okra seed to distinguish between the experimental treatments and not to fully disclose the chemical composition of the seed. Solely, ^{1}H NMR metabolomics was found insufficient to delineate the complete chemical status of a crude sample. Principal component analysis was performed on NMR intensity values and no distinct boundaries, on the principal component analysis plot (Figures 4.8-4.17), could be drawn between the

different treatments. It was therefore concluded that NMR analysis was not sufficient for drawing meaningful biological conclusions.

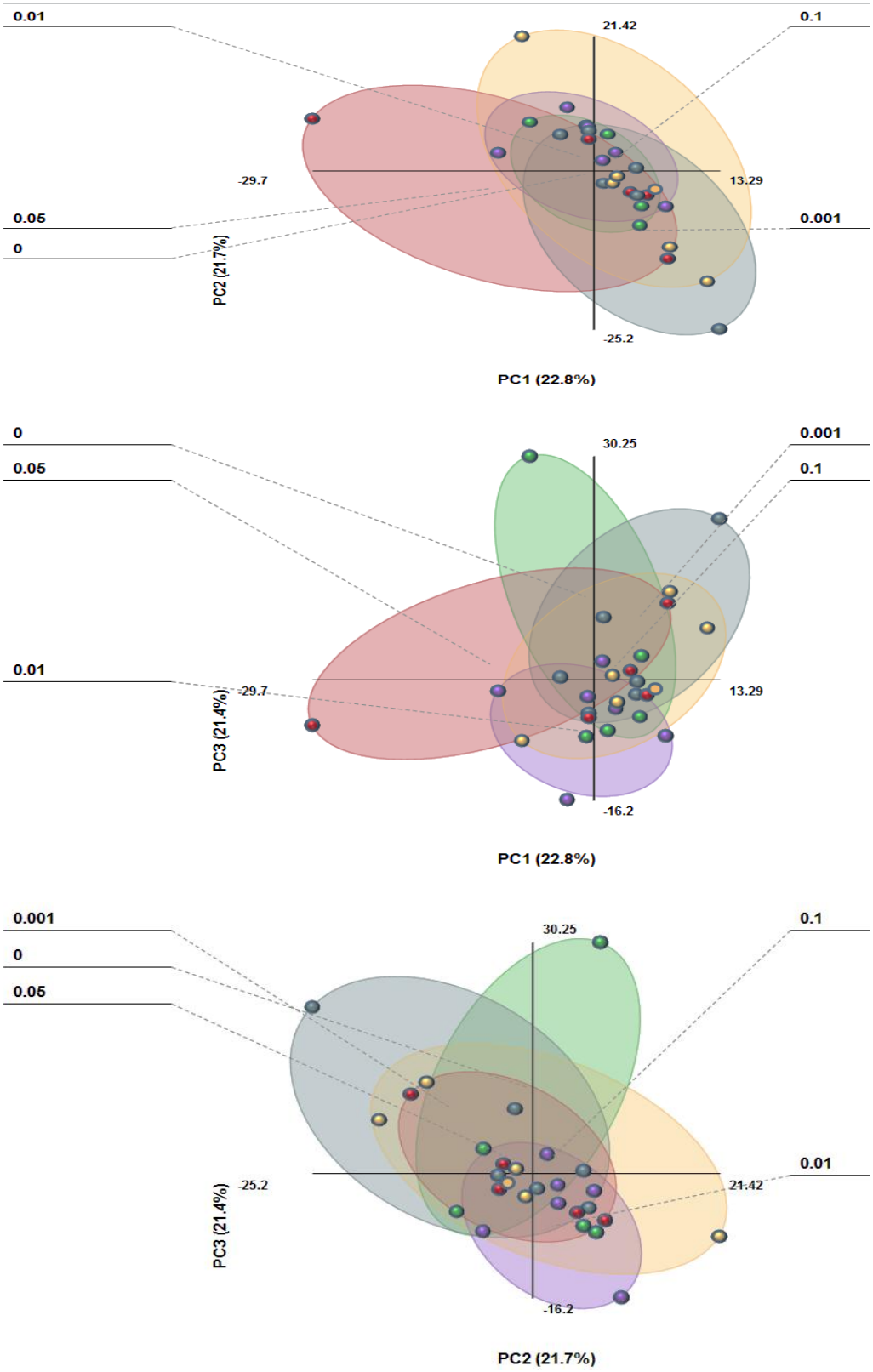

Figure 4.8: PCA for aluminium treatment in 30 samples.

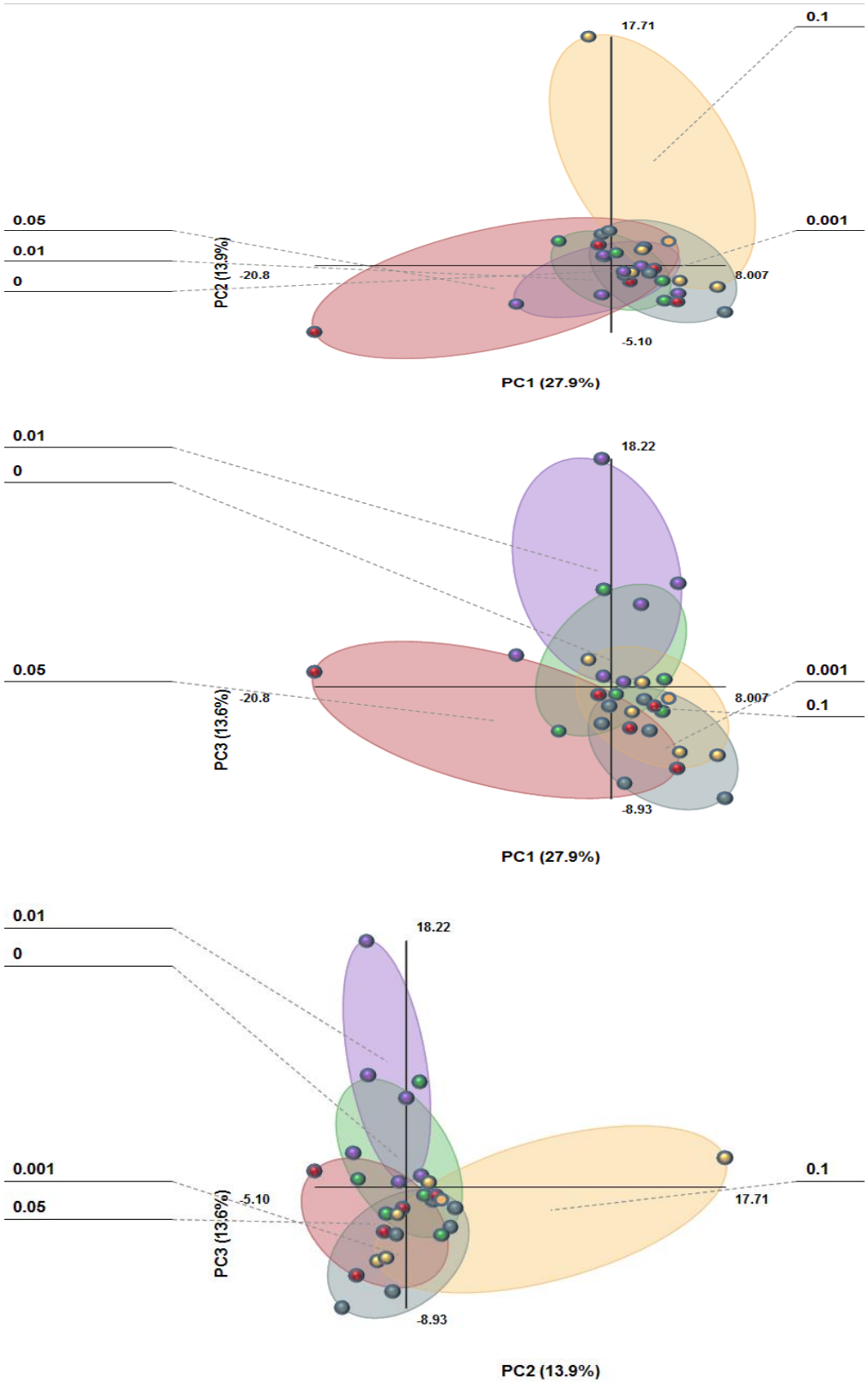

Figure 4.9: PLS-DA for aluminium treatment in 30 samples.

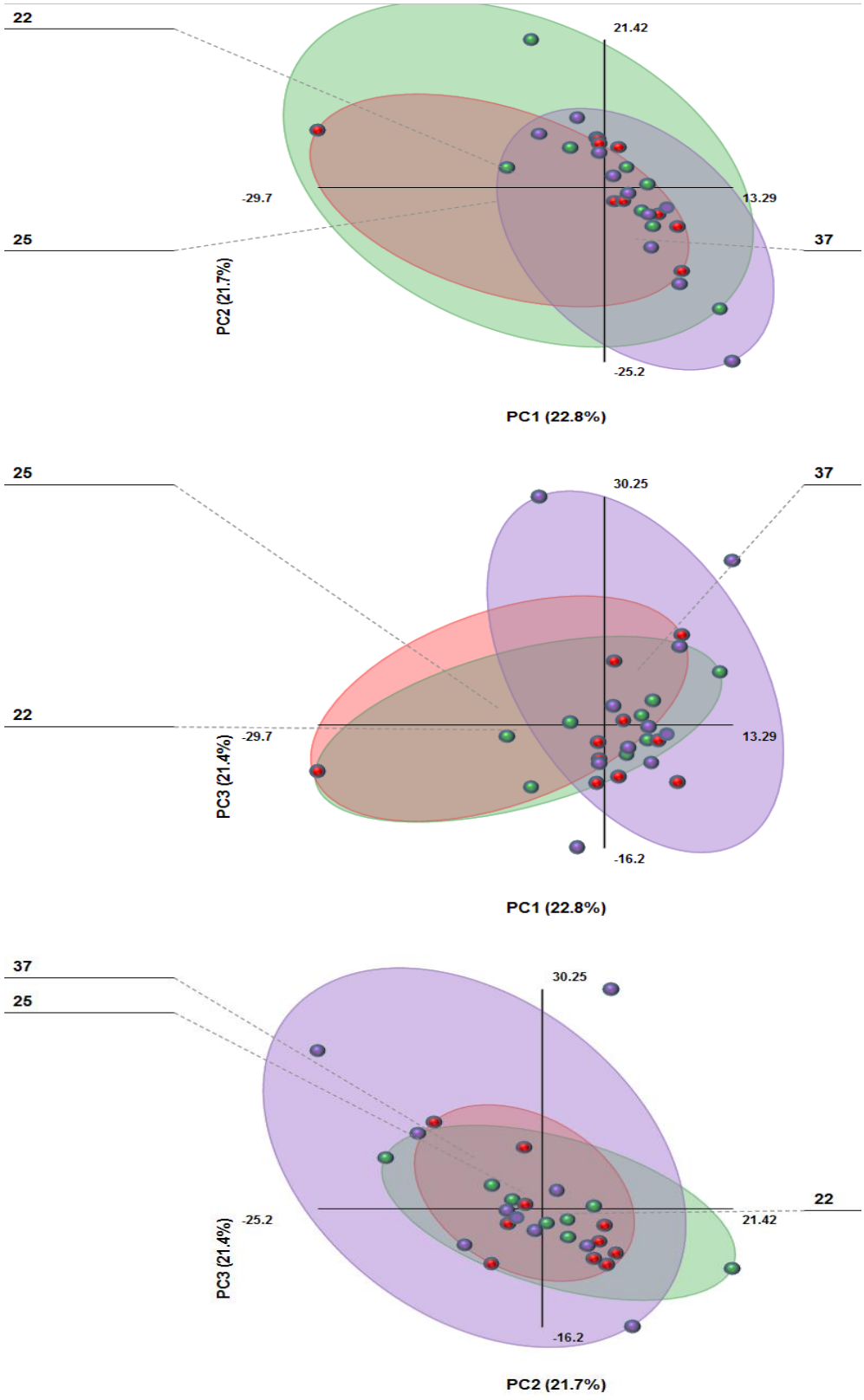

Figure 4.10: PCA for Heat treatment in 30 samples.

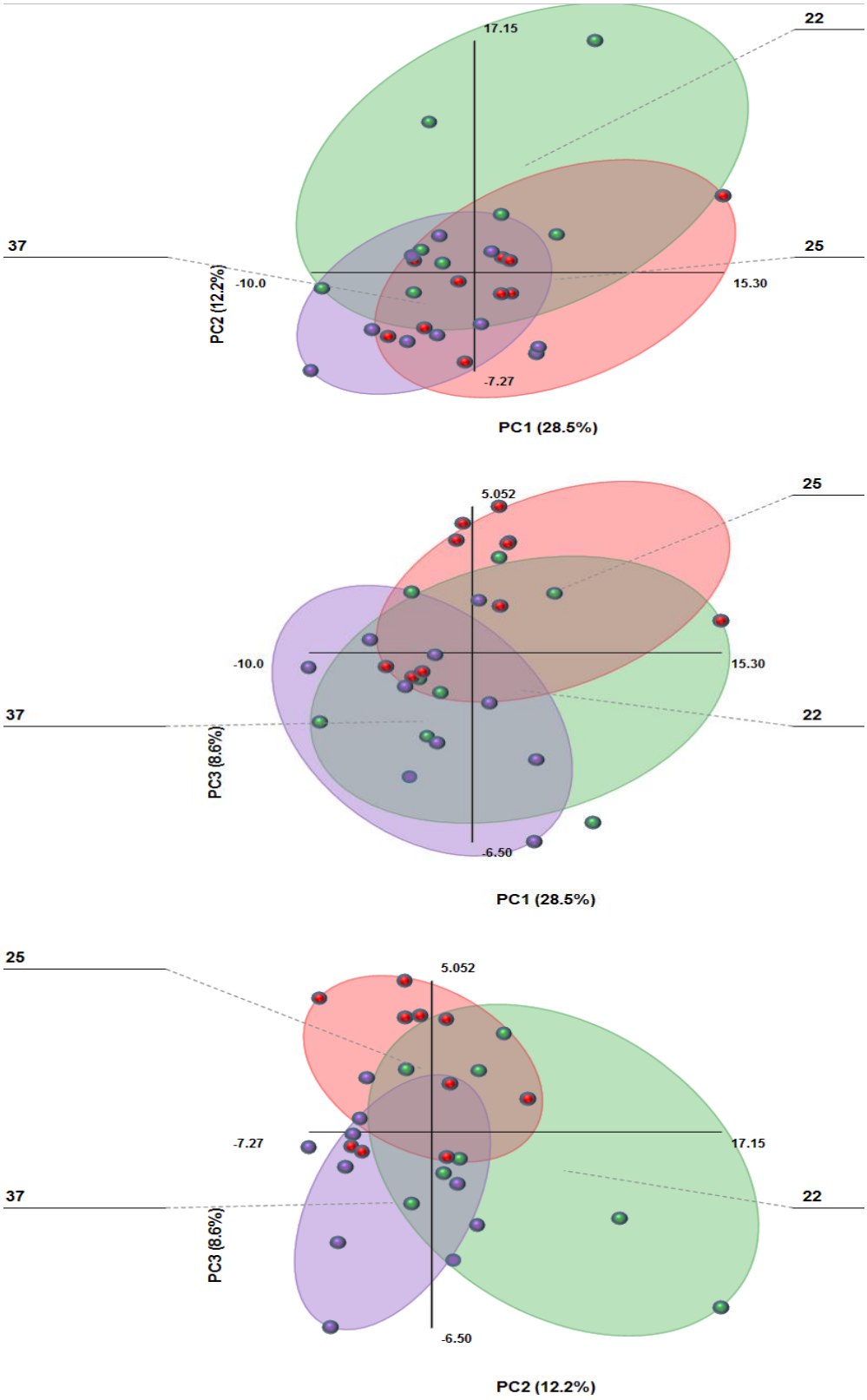

Figure 4.11: PLS-DA for Heat treatment in 30 samples.

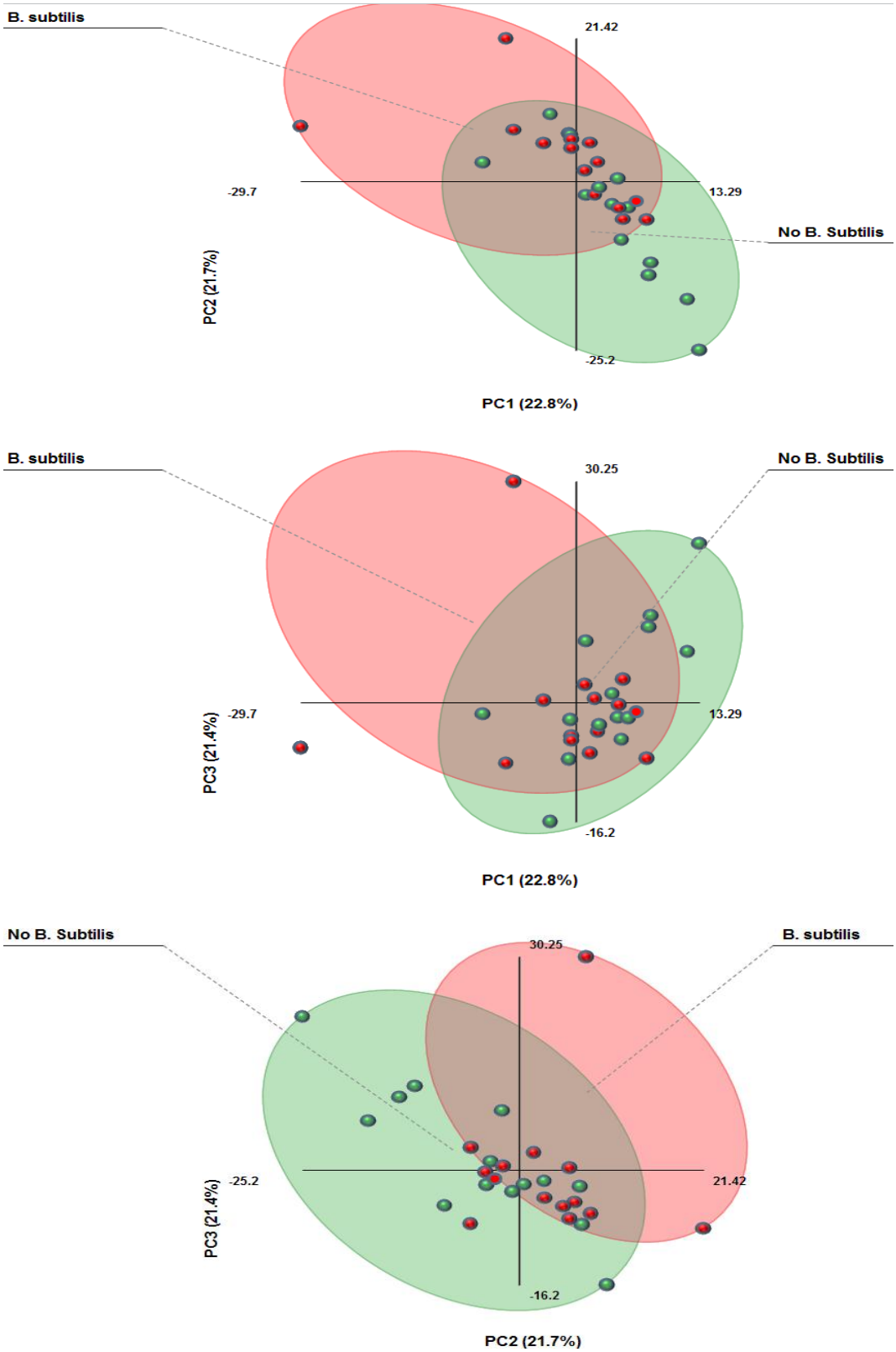

Figure 4.12: PCA for Bacillus treatment in 30 samples in 30 samples.

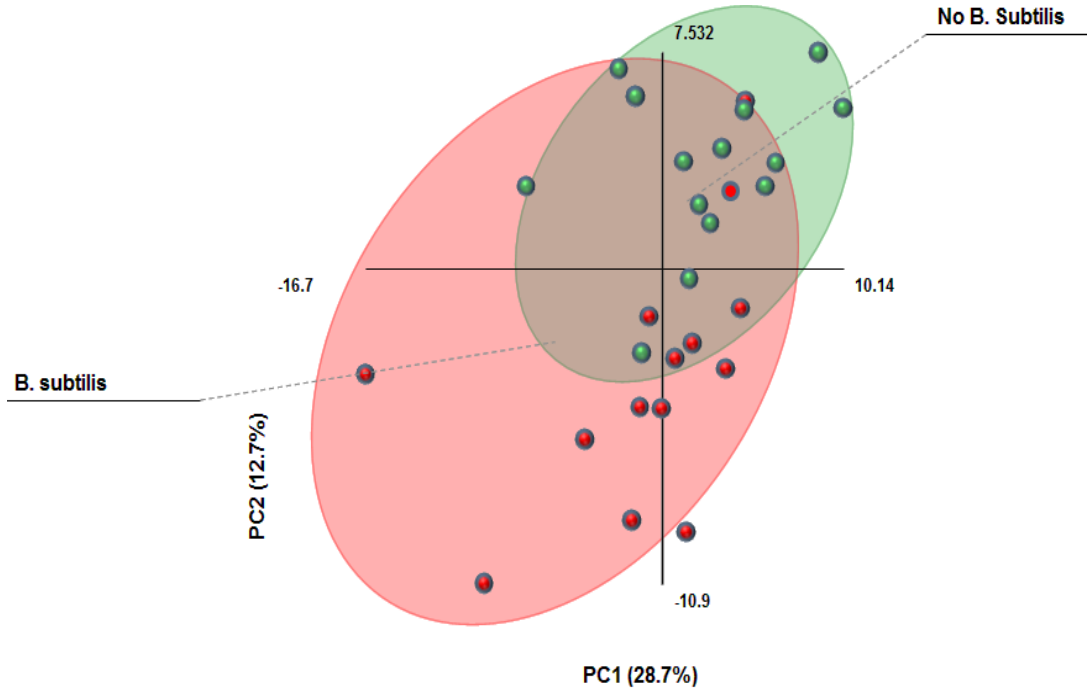

Figure 4.13: PLS-DA for Bacillus treatment in 30 samples.

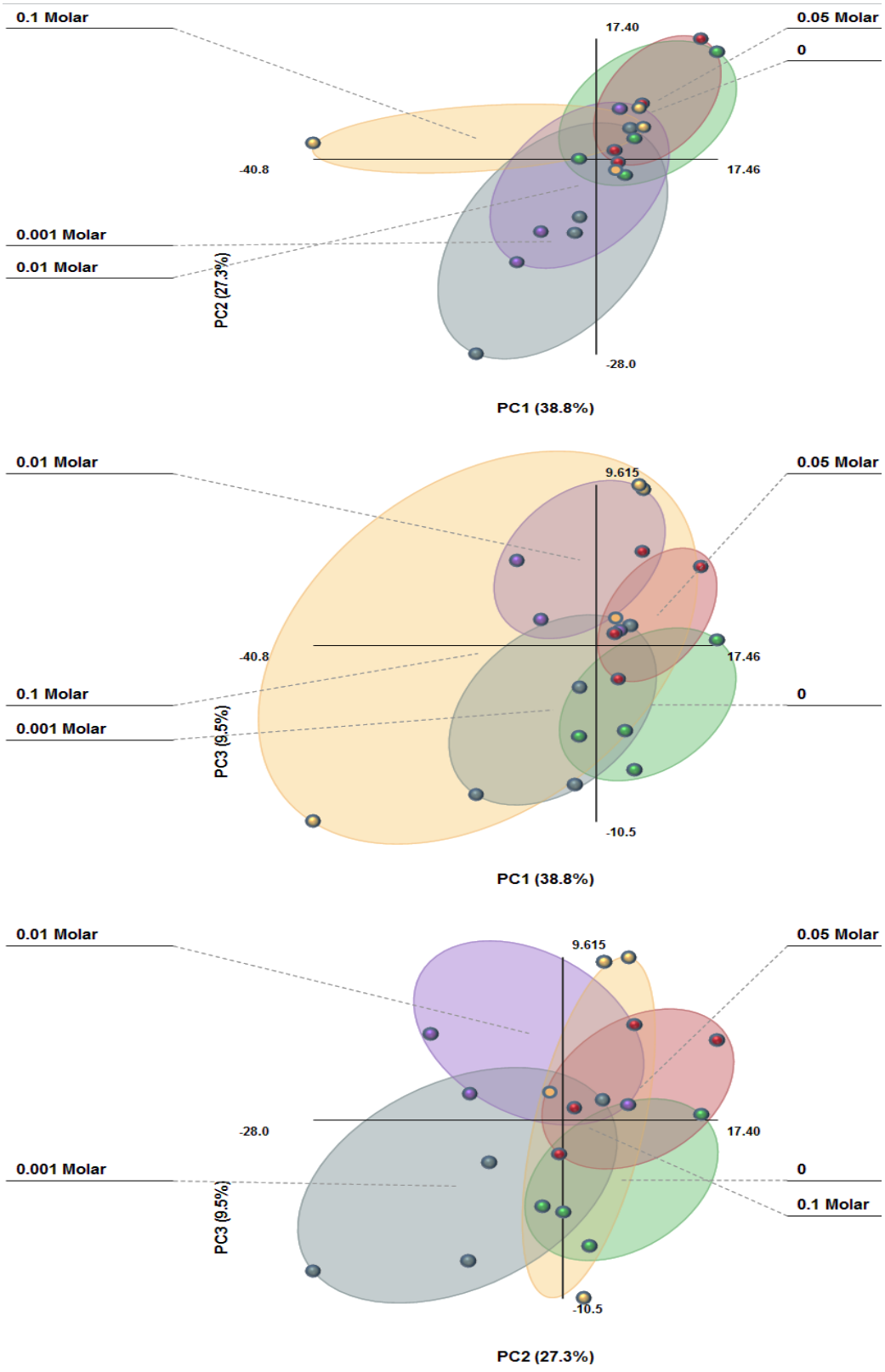

Figure 4.14: PCA for aluminium concentrations in 20 samples.

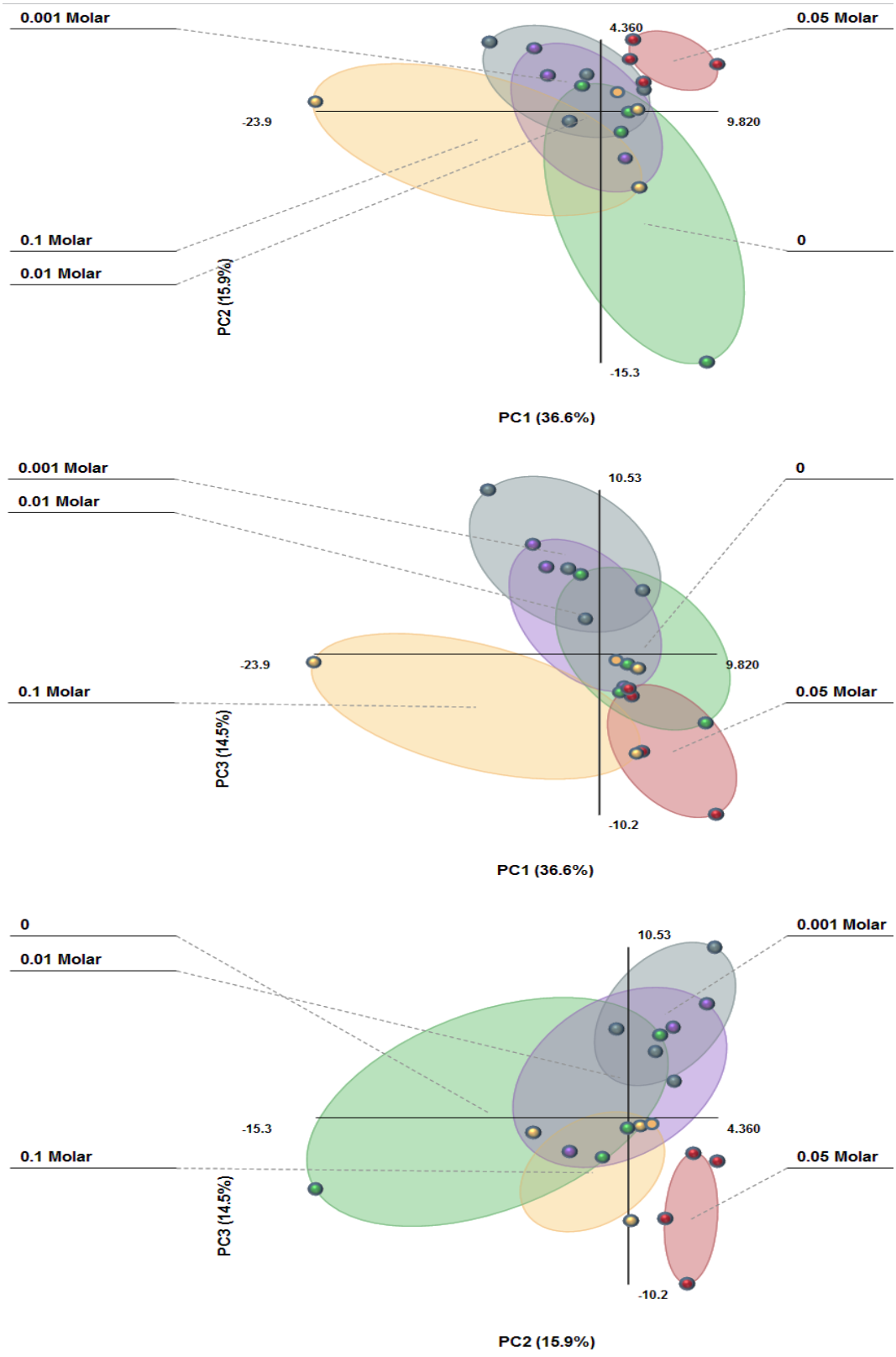

Figure 4.15: PLS-DA for aluminium concentrations in 20 samples.

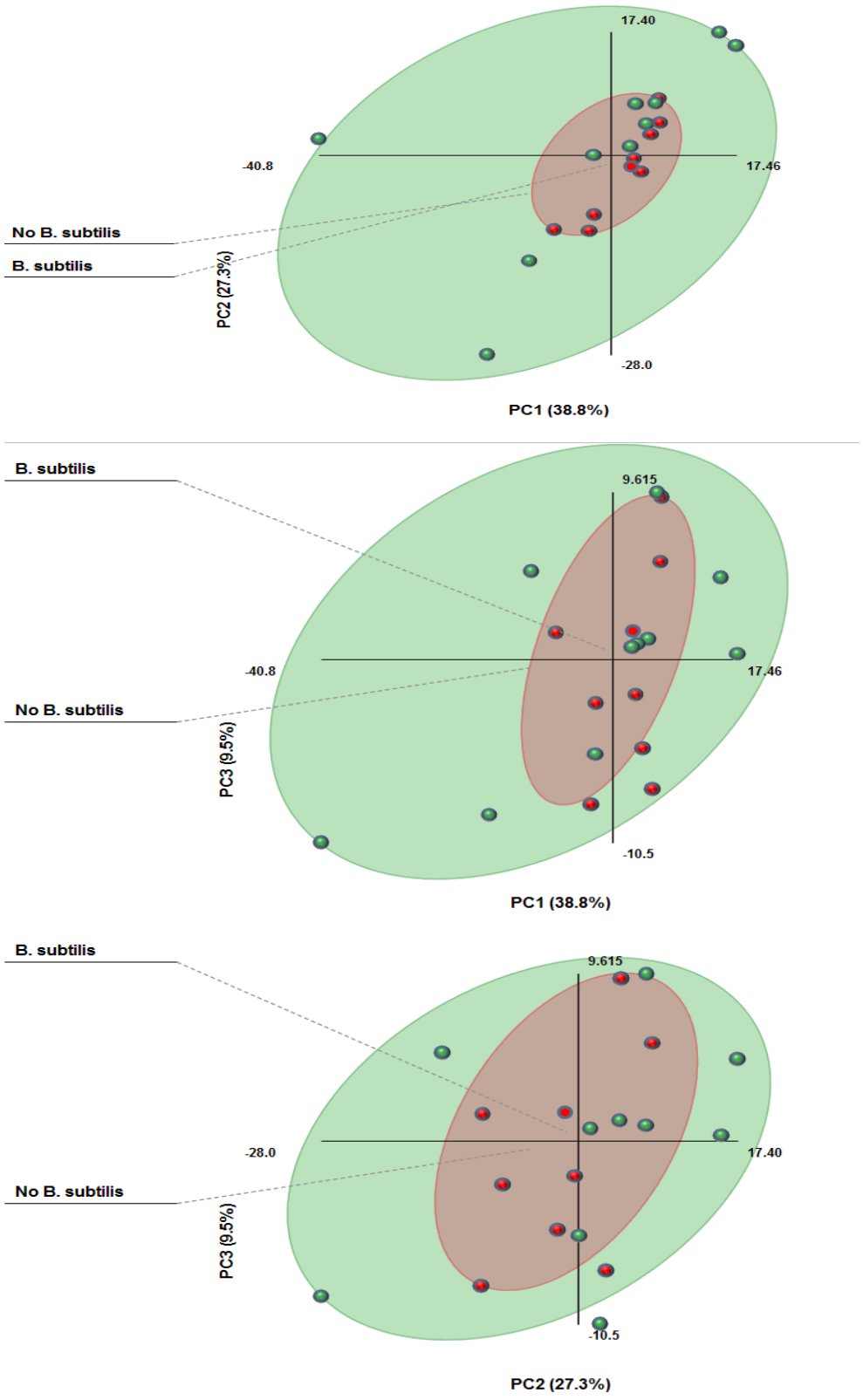

Figure 4.16: PCA for Bacillus treatment in 20 samples.

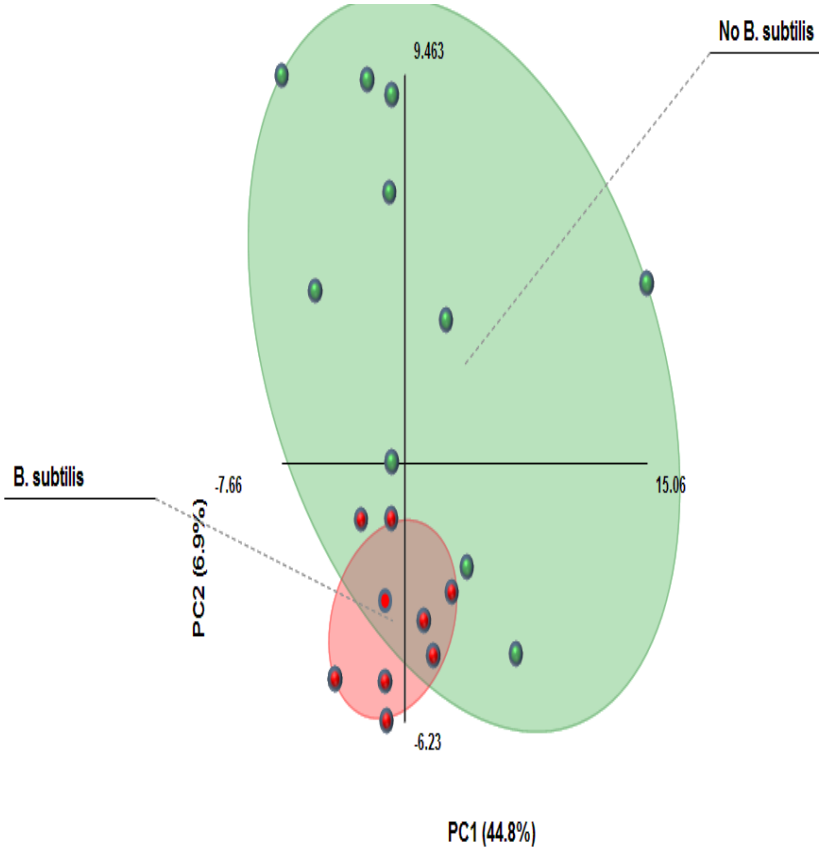

Figure 4.17: PLS-DA for Bacillus treatment in 20 samples.

4.5 Discussion

The study presents for the first time the interaction effects of temperature, aluminium concentration and exposure of the germinating okra seed to *B. subtilis*. Different incubation temperatures, namely, 22°C, 25°C and 37°C where selected based on the results of a preliminary study (Figures 4.1 and 4.2) which showed that 40°C temperature was too hot for germination of okra. For the purposes of this study the interest was on high temperatures because of the increasing need to prospect plant genomes with heat tolerance. Teixeira et al. (2013) found that temperate and sub-tropical agricultural areas may suffer high crop yield loses due to climate change. Because germination influences crop yield, it was therefore necessary to study its influences especially on a significant alternative crop like okra. Different concentrations of aluminium ranging from 0M, 0.001 M, 0.01 M, 0.05 M and 0.1 M

were used to assess their effects on the germination of the okra crop. This concentration range was also selected based on the results of a preliminary investigation (Figures 4.1 and 4.2). Aluminum toxicity threatens plant production and its inclusion in this study was important because attention must not be paid to only heat stress but also to the other stresses which compound the effects of heat stress.

Impenetrable okra seed coat has been showed to cause slow and erratic germination (Demir, 1997; Egley and Elmore, 1987). This form of dormancy interferes with the imbibition and subsequent radicle emergence, oxygen uptake, respiration and homeostasis of endogenous hormones (Mmolawa, 1987). Similar to other dicotyledonous seeds, successful germination of okra seeds can be assessed as firstly; the emergence of radicle which is an embryonic root that will ultimately differentiate into root system and secondly the emergence of hypocotyl which serves as embryonic leaves (two cotyledons). Though studying seeds through quantifying its anatomical changes is simple the recent consensus, it relegates the approach because of its poor explanation of the biochemical changes. For example, evidence showed that germination physiology of seeds starts with the imbibition of water to activate hydrolytic enzymes in biochemical reactions responsible for conversion of reserved starch, proteins and lipids.

In Sionit et al. (1981), the okra cultivar, Clemson Spineless, was reported to germinate within 20/14°C and 23/17°C day/night temperature range, while 17/11°C temperature inhibited emergence of primary leaves.

Coating of the seed with *B. subtilis* could only be done in two treatments to assess the possibility if such a common soil inhabitant could be used as a seed dressing. The aspect of coating with *B. subtilis* can be combined with the various priming methods which increased seed germination, seedling vigour, mean germination time and marketable fruit yield in okra in the study of Sharma et al. (2013). Other than enhancing germination and seedling vigour, *Bacillus* species increase plant growth by producing antibiotics (Awais et al., 2007) and toxins (Mukry et al., 2010) to compete with plant pathogenic bacteria, and are biological control agents against wilt disease of pigeonpea (Siddiqui and Manmood, 1995) among other plant diseases.

It is well known that when seedlings are exposed to temperatures higher than the thresholds for optimal growth, heat shock proteins are produced at the expense of the production of normal proteins (Key et al., 1985a,b,c; Nagao et al., 1986; Neuman

et al., 1989; Nover and Scharf, 1997). Because of difficulties in delineating proteins in response to stress this study focused on the metabolome because the cascading effects of plant stress causes instant metabolic shifts. This would be applicable not only to temperature but also to aluminium toxicity and also the altered environment with the presence of *B. subtilis* as seed coat treatment. Using ^{1}H NMR metabolomic approaches, experimental treatments have successfully imposed influenced changes in the resultant metabolome of the okra seed, with rather a distinct set of a shared metabolome.

The scarcity or lack of similar studies limited the investigation on the metabolic transitions on the germination of okra in response to the treatments in this study. However, other methods for analysing ^{1}H NMR data e.g. the partial least square-enhanced discriminant analysis could segregate between treatments therefore allowing meaningful biological assumptions to be made. However, not much variation was accounted for when partial least square-enhanced discriminant analysis was applied. It is recognized that this study had some shortfalls. Only one variety of okra, namely, Clemson Spineless, was used to access the influence of temperature, aluminium toxicity and exposure to *B. subtilis* on the growth of this plant. A plethora of studies show that, as in the study of Dawood et al. (2013), that chemical compositions during germination of canola are variety dependent. This study has therefore missed the opportunity to investigate the effects of genotype on the dynamics of germination of okra. Moreover, Ou-yang et al. (2014) found genotype differences when investigating the levels of aluminium tolerance in *Jatropha curcas*. The time course of the germination assays was short, five days, and therefore no conclusions could be made on the development of the seedling.

The influences of treatments on ionome and metabolome of germinated seeds were assessed through multivariate analytical tools with the main objective to show shifts in general metabolism, as cofounded with treatment variations. In general, principal component analysis (applied on NMR intensity values) was not able to discriminate, with clear boundaries, the differences between treatments, and therefore no meaningful conclusions could be drawn to explain biological processes. . In a situation whereby the ^{1}H NMR metabolomic fingerprinting is insufficient to discriminate between treatments (as was the situation with this study), it is recommended that further analysis is performed to delineate the chemical make-up

of a samples. However, it was possible to deduce from the NMR analysis that temperature, concentration of aluminium in the germination medium and exposure of the germinating seed to *B. subtilis* probably influence just a small proportion of the chemistry of the okra. It is evident from the lack of clear-cut discrimination of the treatments by principal component analysis of the NMR intensity values. It is recommended that follow-up studies consider the critical areas not attended to during the course of this study.

As one of the omics technologies ^{1}H NMR is renowned for its potential to discern an array of metabolites expressed during plant growth and development.

CHAPTER 5

5. FUTURE RESEARCH AND CONCLUSIONS

5.1 Thermotolerance

Different temperatures were used to determine the most critical level at which germination is most inhibited. The least germination percentages and coleoptile lengths were observed in 37°C in contrast to 22°C and 25°C temperature. The results testified that high temperatures inhibit progress in germination, and 25°C is the optimum temperature for germination physiology.

5.2 Aluminium tolerance and its potential as a seed priming treatment

The study made use of various aluminium concentrations with the view to suggest threshold with greater inhibition of okra seed germination. Five concentrations, 0M, 0.001 M, 0.01 M, 0.05 M and 0.1 M, were used. The results showed that germination percentages were invariably inhibited at 0.1 M, despite temperature level or bacterial coating treatment. Furthermore, 0.001 M concentration stimulated both germination percentages and coleoptile lengths. However, inclusion of *Bacillus subtilis* treatment on tested seed showed widening of stimulation effects in cumulative germination percentages and growth vigour, confirming that aluminium has the potential as a seed treatment to invigorate seed germination.

5.3 Ameliorative effects of beneficial bacteria in seed germination stress

Bacterial coating treatment showed improved growth in germination percentages and coleoptile lengths of okra seed in 25°C temperature. In this temperature there was growth stimulation at 0.001 M, 0.01 M and 0.05 M concentrations, unlike other temperatures. However, high temperature and aluminium concentration suppressed biological effects of the bacterium to ameliorate physiological constraints imposed by aluminium ions and temperature. Results of the study showed that bacterial coating treatment was unable to stimulate both germination percentages and coleoptile lengths in 0.1 M aluminium concentration and 37°C temperature.

5.4 Interaction effects: Aluminium concentrations, bacterial seed treatment and germination period

Factorial interaction is important to understand the biology of multitrophic interaction encountered in the study. In the study these terms were treated as sources of variation in the biology of okra seed germination. Relative to 22°C and 37°C, 25°C temperature treatment had the highest contribution in the following interactions, *viz*, (1) Aluminium concentrations x Bacterial seed treatment, (2) Aluminium concentrations x Germination period, (3) Bacterial seed treatment x Germination period and (4) Aluminium concentrations x Bacterial seed treatment x Germination period, for germination percentages. As for coleoptile lengths similar to germination percentages, 25°C temperature also encouraged growth.

5.5 Chemistries of treatments

Treatment of the seed with *B. subtilis* was expected to raise the quanties of different chemical elements. However, the chemical contribution by the bacterial treatment was found to be negligible.

5. 6 ^{1}H NMR metabolomics association

Metabolic associations as visualized from biplots of principal component analysis performed on NMR intensity values showed no discreet grouping of expressed metabolites. However, in all the associations metabolic clusters expressed a sharing of a subset of metabolites. It was therefore concluded that the treatments had very insignificant effects on the gross physiology of the germinating okra seed.

5.7 Significance of the findings

Integrating phenotype data with high throughput data from technologies such ^{1}H NMR and comparable ones will aid in understanding the biological responses of neglected crops such as okra. Unlike solely using traditional techniques, the approach is even greatly encouraged because of its potential to elucidate underlying

biological functions responsible for adjudicating multitrophic interations in plants. Thus, application of omics technologies such as NMR and equivalent will be able to fast-track improvement of neglected okra. Hardier crop plants with greater economic potential are much sort after in diversifying cropping systems in South Africa.

5.8 Future research

With the expanse of the study having anticipated the quest to unravel physiological responses in okra showing novelty in plant biological research, it is more than worthy to substantiate the deficiencies encountered thereof. Principal to the whole account is that the study was intentionally made to focus on okra seed biology given the challenges encountered in cultivations, as reviewed through literature. Earlier research accounts attributed inconsistent germination responses to temperature variations and seed factors. The latter factors were addressed through commercial seed production systems

Firstly, metabolic associations in germinating okra seeds showed no discreet grouping amongs treatments. This might be inferred as indicating that phenotypic responses encountered in seed germination are related to overlapping metabolic pathways. Since the study was successful to determine baseline information, it will be necessary to identify an array of those molecular compounds expressing the combination effects of heat and aluminium stresses in seed germination. Given that temperature tends to facilitate diffusivity of metal ions from the germination environment, it is imperative to determine the effects of the two factors on ultra-structural development of the seeds during germination in conditions of reduced water potential. Secondly, promoting uniform germination through the use of treatments such as those important in reducing resistance of seed coats is suggested. In this study 0.001 M aluminium concentration showed stimulative effects on germination percentages and coleoptile lengths, therefore suggesting that has a role to play in eliminating imbibition factors which are primary in seed germination. Also, it will be important to further evaluate the biological effects of the concentration in further seedlings development of the plant. Thirdly, the study suggests further determination of colony behaviour of *B. subtilis* in bioremediating seed germination biology. In the current study it is evident that bacterial coating treatment of seeds

invariably stimulated germination and vigour of the seeds in 22°C and 25°C than in 37°C. Also, the bacterium was able to reduce the effects of effects of aluminium ions in all concentrations than in 0.1 M, which suggest further studies to determine thresholds of aluminium ions in the bacterium.

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